ZO-1 DIRECTS ELECTRICAL SYNAPSE FORMATION IN THE CNS OF DEVELOPING ZEBRAFISH.

by

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A DISSERTATION

Presented to the Department of Biology
and the Division of Graduate Studies of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

June 2021

DISSERTATION APPROVAL PAGE

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Title: ZO-1 Directs Electrical Synapse Formation In The CNS Of Developing Zebrafish.

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DISSERTATION ABSTRACT

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Doctor of Philosophy

Department of Biology

June 2021

Title: ZO-1 Directs Electrical Synapse Formation In The CNS Of Developing Zebrafish.

Electrical synapses are specialized cellular adhesions that allow for the direct flow of information between neurons. They are required for proper neural circuit development and make up an estimated 20% of synapses in adult nervous systems. Despite this, we know very little about their molecular structure. We often consider them to simply be aggregates of Connexin protein channels, and, up until now, it has been unclear whether electrical synapses require other support proteins for them to form and function. In this thesis, I show that not only do electrical synapses require the support of another molecule, but their formation is actually dictated by it. A comparison of electrical synapses to other cellular adhesions led me to hypothesize that they would require an intracellular scaffolding molecule to instruct their formation. Using forward and reverse genetics, the scaffolding protein Zonula occludens-1 (ZO1) was identified as being critical for Connexin protein localization to the electrical synapse. Further, I showed that its organizational role at the synapse is mediated by a direct interaction with the postsynaptic Connexin. The presence and requirement of ZO1 at the electrical synapse forces us to update our simple model and shows that electrical synapses are complex structures that require molecular organization beyond the channel-forming Connexin proteins. This dissertation contains previously published and co-authored material.

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Lasseigne AM*, Echeverry F*, Ijaz S*, Michel JC*, Martin EA, Marsh AJ, Trujillo E, Marsden KC, Pereda AE, Miller AC (2021). Electrical synaptic transmission requires a postsynaptic scaffolding protein. eLife, 10:e66898 DOI: 10.7554/eLife.668.

Martin EA*, Lasseigne AM* and Miller AC (2020). Understanding the molecular and cell biological mechanisms of electrical synapse formation. Frontiers in Neuroanatomy. 14:12.

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ACKNOWLEDGMENTS

I would first like to thank Adam Miller for his guidance, patience, and kindness over the past five years. I am honored to have been your first graduate student, and I look forward to what you are able to accomplish in the future. Thank you for growing with me and always having my back. I would also like to thank the Miller lab, my cohort, Womxn in Neuroscience, my DnD crew, my friends, and anyone who ever brought me coffee or chocolate when I was having a bad day. And, finally, I would like to thank my parents, Lori and Jon, my little brother, Nick, and my partner, John Britton. I could not have done this without you.

This work was supported in part by the Developmental Biology Training Grant, T32HD007348.

To my parents, Lori and Jon.
Thank you for everything.

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CHAPTER I

INTRODUCTION

Think for a moment about picking up a cup. To pick up this cup, you need to see the cup. Light information from your surroundings must be detected by your retina and then interpreted by your brain. Next, your brain needs to tell the muscles of your arm and hand to reach out to the location of the cup, grasp it, and then lift it with the appropriate amount of force. While this action is occurring, your body needs to recognize your position in space and adjust as your weight shifts and as you feel the weight of the cup. Simultaneously, you may be thinking about why you are picking the cup up in the first place. Are you thirsty? Did someone ask you to pick up the cup? Do you recognize the cup? This simultaneous processing of sensory information, control over muscles, decision making, and recall of memory is occurring in your brain almost instantaneously, in addition to the constant general functioning required to keep you alive.

The human brain integrates and processes massive amounts of information, all encoded by electrical impulses passed between specialized cells called neurons. Humans have an estimated 86 billion neurons (Azevedo et al., 2009), all containing identical biological instructions (i.e., DNA) but resulting in a diverse population of cells. Each of these neurons then makes thousands of connections, called synapses, to other neurons creating a biological processing network. Synapses are critical to the nervous systems ability to move and process information, and malfunctions in synapse function contribute to a wide variety of neurological disorders in humans (Grant, 2019). The field of neuroscience, therefore, aims to determine how the biological instructions contained within a neuron can lead to cellular and synaptic changes that ultimately impact behavior.

To this end, one synaptic subtype, the chemical synapse, has been the focus of neurobiological research for decades. At chemical synapses, electrical signals in one neuron trigger the release of chemical neurotransmitters across a physical gap that then interact with receptors on the receiving cell. Over the years, we have learned that these structures require expansive, asymmetric networks of molecular machinery (Ackermann et al., 2015; Grant, 2019; Siddiqui and Craig, 2011). However, the field's focus on the

chemical synapse has led it to largely neglect the second synaptic subtype: the electrical synapse.

Electrical synapses are unique from their chemical counterparts in that they physically link the cytoplasm of adjacent neurons via Connexin (Cx) protein channels. This allows the electrical signal itself to directly move from one cell to another producing a faster, more energy efficient, and often bidirectional mode of communication (Nagy et al., 2018). Electrical synapses are best known for their role in developing circuits, where they can act as transient precursors for permanent chemical synapses and are critical for proper circuit formation in many cases (Zolnik & Connors, 2016; Maher et al., 2009). Recently, it has also been shown that electrical synapses are prevalent in adult circuits. Work in the rodent retina and *C. elegans* have estimated that 20% of the synapses in mature circuits might be electrical (White et al., 1986; Anderson et al., 2011; Jarrell et al., 2012; Cook et al., 2019). Despite their functions in both developing and mature circuits, little is understood about how these structures actually develop. Electrical synapses are often viewed as simplistic aggregates of Connexin channels, but their structural diversity and functional regulation alone contradict such a simple model (Nagy et al., 2018; Pereda, 2016; O'Brien, 2017). Furthermore, their unique functional properties impact neuronal circuits in ways that chemical synapses cannot (Hormuzdi et al., 2004; Connors, 2017). Therefore, for us to have a complete understanding of the nervous system, it is critical that we investigate the molecular mechanisms underlying electrical synapse formation and function.

Electrical synapses are gap junctions found between neurons. Each channel is composed of two Connexin hexamer hemichannels: one presented from each of the adjoining cells. Therefore, each individual channel is made up of 12 Connexin proteins, and each electrical synapse is made up of tens to thousands of these channels (Goodenough and Paul, 2009). But how do the Connexins get to the synapse? How do they know where to go? How are these structures maintained over time? The mechanisms required for Connexin localization and function are not understood. However, there is growing evidence of molecular complexity at the electrical synapse that could have a role in building these structures. First, electron microscopy images depict an electron dense region surrounding the Connexin plaques, indicating a concentration of proteins beyond

the gap junction channels (Llinas et al. 1974). Second, electrical synapses are diverse structures found in stereotyped arrangements (Nagy et al., 2018; Connors, 2017). This requires a level of organization that must be robust enough to recruit in necessary materials between precise partners but also flexible enough to allow diversity. And third, electrical synapses are highly dynamic. Neuronal Connexin proteins themselves are turned over about every three hours, requiring: the constant trafficking of new Connexins, exocytosis machinery to embed them in the membrane, and endocytosis machinery to clear old channels (Lauf et al., 2002; Flores et al., 2012; Wang et al., 2015). Additionally, electrical synapses are highly regulated, and their strength can be modified by changes in cell electrical state, channel abundance, or the number of channels that are open (O'Brien, 2017; Pereda, 2016). These properties of electrical synapses suggest that there must be underlying molecular support beyond the Connexin channels.

In this thesis, I identify and characterize one of these necessary proteins. In Chapter II, to investigate the molecular support network that might underly electrical synapse function, I review the literature on cellular adhesions comparing electrical synapses to chemical synapses, tight junctions, adherens junctions, and non-neuronal gap junctions. I use these comparisons to generate hypotheses as to how electrical synapses might be established, how neuronal connexins might be recruited and trafficked to synapses, and what proteins or types of proteins are most likely necessary at electrical synapses. Ultimately, this review leads me to hypothesize that an intracellular scaffolding molecule will be required for synapse formation and function. In Chapter III, I use the zebrafish central nervous system as a model to identify and characterize the role of a required scaffolding protein, Zonula occludens-1b, at electrical synapses. In chapter IV, I investigate the cell biology of this scaffold and conclude that it functions exclusively in the postsynaptic compartment of the neuron via a direct interaction with the channel forming Connexins. Altogether, this work identifies and defines the functional role of a protein that dictates the structure and function of electrical synapses. This dissertation contains previously published and co- authored material.

CHAPTER II

UNDERSTANDING THE MOLECULAR AND CELL BIOLOGICAL MECHANISMS OF ELECTRICAL SYNAPSE FORMATION

Martin EA*, Lasseigne AM* and Miller AC (2020). *Front. Neuroanat. 14*:12.
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Author Contributions

EM, AL, and AM discussed and wrote the review. All authors contributed to manuscript revision, read and approved the submitted version.

Introduction

Electrical synapses are specialized connections between neurons that facilitate direct ionic and small metabolite communication (Fig. 1). They are composed of tens to thousands of gap junction channels clustered together into plaques that are present throughout developing and adult brains. Electrical synapses contribute towards initial neural circuit function including driving the earliest animal behaviors (Rekling et al., 2000; Saint-Amant and Drapeau, 2000; Marin-Burgin et al., 2006; Su et al., 2017) and continue to function broadly throughout life in neural circuits controlling sensory processing (Li et al., 2009; Huang et al., 2010; Yaksi and Wilson, 2010; Pouille et al., 2017), rhythmic behavior in central pattern generators and motor systems (Eisen and Marder, 1982; Song et al., 2016; Traub et al., 2020), and cortical processing in mammals (Galarreta and Hestrin, 2001, 2002; Connors and Long, 2004; Gibson et al., 2005; Hestrin and Galarreta, 2005; Mancilla et al., 2007). Despite these well-documented and diverse circuit functions (reviewed in Nagy et al., 2018), the electrical synapse is commonly thought of as a necessary, but simple and temporary, precursor in development to the later-forming chemical synapse. However, emerging evidence supports an alternative view, namely that electrical and chemical synapses are essential life-long collaborators in

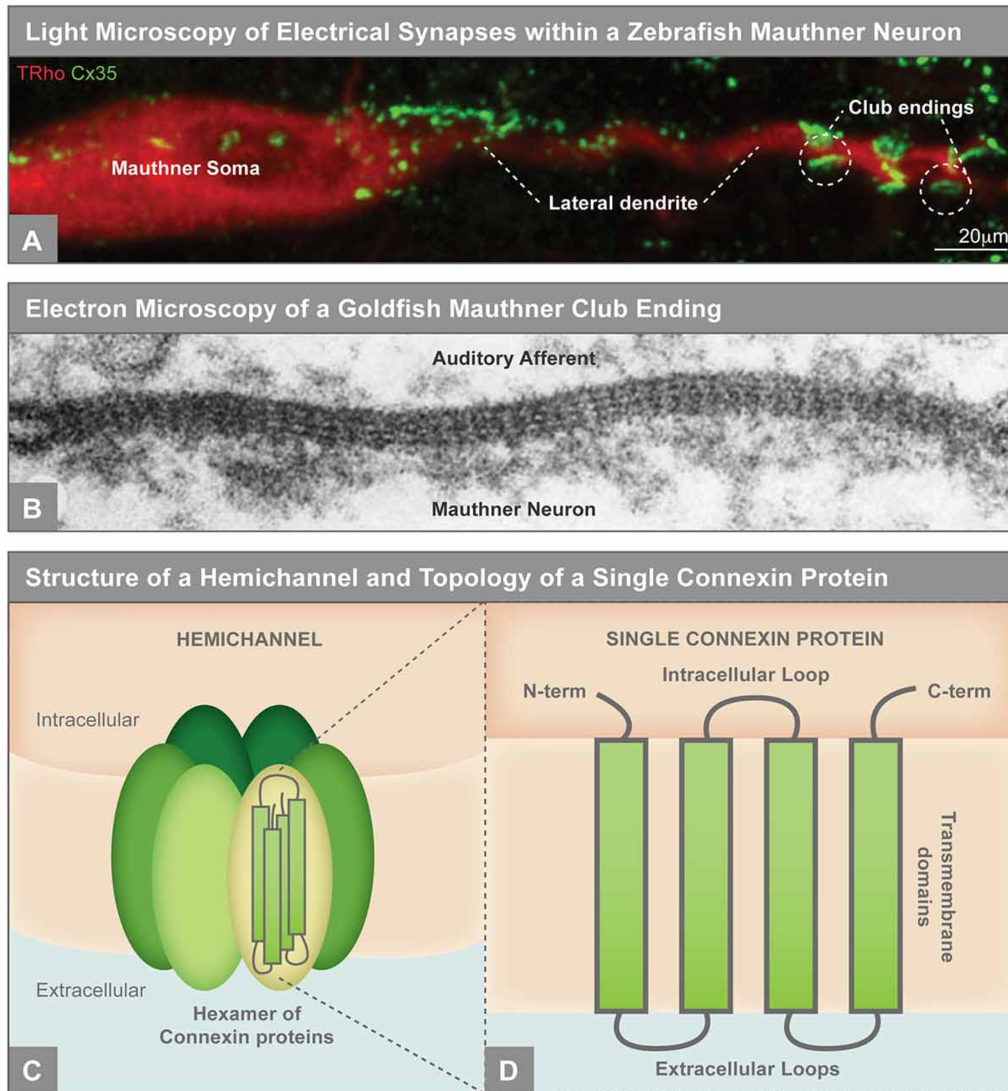


Figure 1. (A) Electrical synapses visualized by light microscopy on the larval zebrafish Mauthner neuron. Mauthner, labeled with tetramethylrhodamine-dextran (TRho, red), makes electrical synapses, labeled by Connexin35 (Cx35, green), on its soma and lateral dendrite. The so-called club ending synapses represent uniquely identifiable electrical connections with auditory afferents. The Mauthner neuron has served as a key model for electrical synapse formation and function and the principles learned have applied to both invertebrate and vertebrate systems (Nagy et al., 2018). Image modified from Yao et al. (2014), reproduced with permission. (B) Electron microscopy showing gap junctions at the club endings between the postsynaptic Mauthner neuron and the presynaptic auditory afferents in adult goldfish. The electron density between the neurons and the characteristic intermembrane spacing are hallmarks of gap junctions. X 285,000. Republished with permission of Rockefeller University Press, from Brightman and Reese (1969); permission conveyed through Copyright Clearance Center, Inc. (C) Illustration of an unpaired gap junction hemichannel inserted into the plasma membrane, composed of a hexamer of Connexin proteins. (D) A single Connexin protein is illustrated to show protein topology.

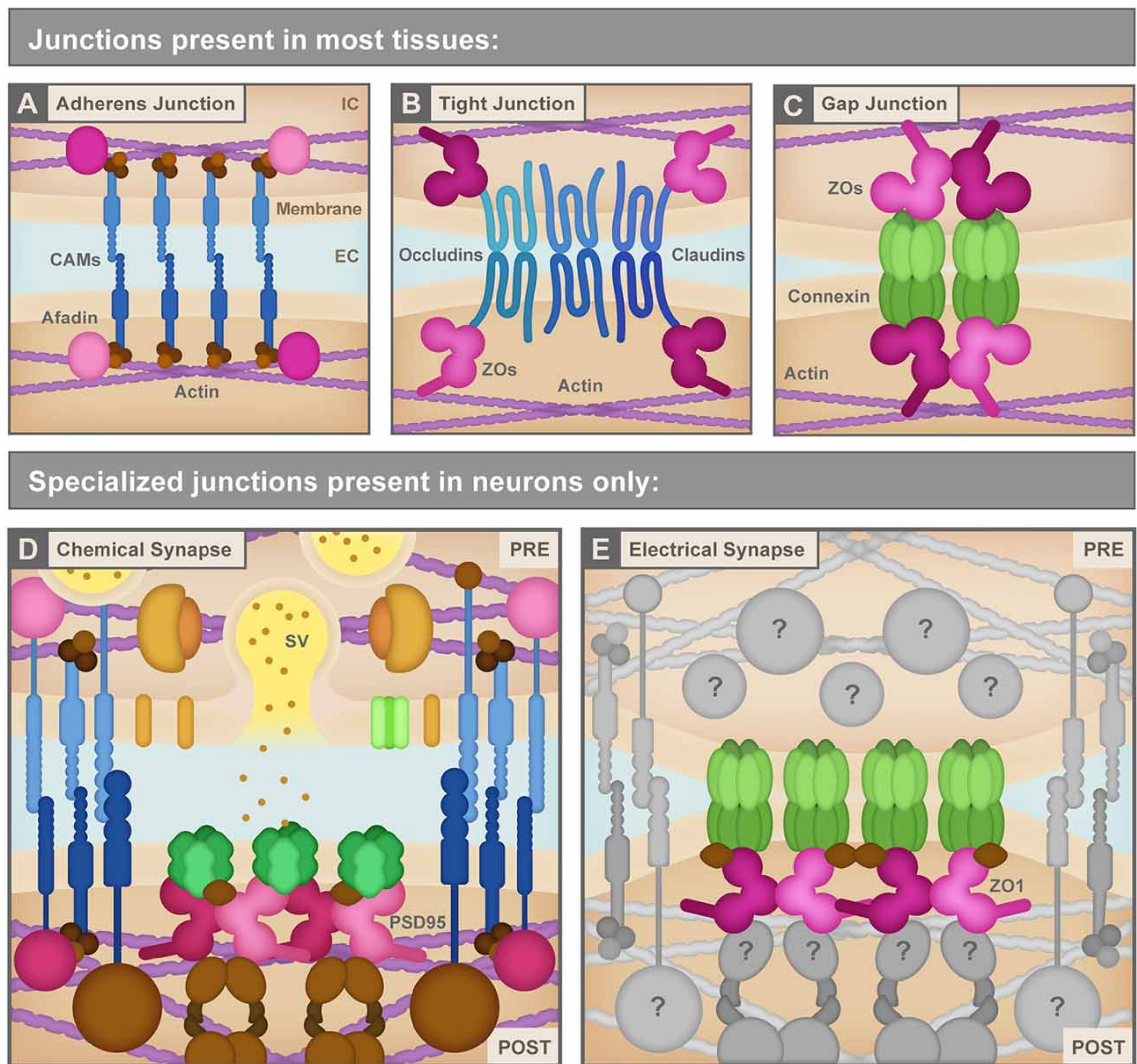
dynamically shape brain function (reviewed extensively in Pereda, 2014). Indeed, the best-studied electron-microscope reconstructed connectomes, of *C. elegans* and the rabbit retina, reveal that electrical synapses make up about 20% of connections in these mature circuits (White et al., 1986; Anderson et al., 2011; Jarrell et al., 2012; Cook et al., 2019).

Also, electrical synapses have emerged as complex biochemical structures, with their proteomic diversity supporting sophisticated neuronal functions including activity-dependent plasticity (reviewed in Miller and Pereda, 2017). These findings lead to exciting new ideas about the role of electrical synapses in brain development, function, and disease. However, while abundant literature has explored the mechanisms that build both non-neuronal gap junction and chemical synapse formation, the field still has only furtive glances into the cell biological mechanisms that control electrical synapse formation and function. Given that electrical synapses are formed within the elaborate architecture of neurons and that they are optimized for fast transmission and plasticity, we expect that complex cell biological rules regulate the formation and homeostasis of these gap junction channels. Here we focus on emerging evidence that provides the first glimpse of electrical synapse cell biology *in vivo*. We apologize for the many excellent articles we were unable to cite in this review due to space constraints, but the explosion of renewed interest in these structures has generated many recent reviews that provide excellent resources to examine the many aspects of electrical synapse structure and function (Dong et al., 2018; Harris, 2018; Jabeen and Thirumalai, 2018; O'Brien and Bloomfield, 2018; Traub et al., 2018; Alcamí and Pereda, 2019; Totland et al., 2020). We expect that complex cell biological rules regulate the formation and homeostasis of these gap junction channels. Here we focus on emerging evidence that provides the first glimpse of electrical synapse cell biology *in vivo*. We apologize for the many excellent articles we were unable to cite in this review due to space constraints, but the explosion of renewed interest in these structures has generated many recent reviews that provide excellent resources to examine the many aspects of electrical synapse structure and function (Dong et al., 2018; Harris, 2018; Jabeen and Thirumalai, 2018; O'Brien and Bloomfield, 2018; Traub et al., 2018; Alcamí and Pereda, 2019; Totland et al., 2020).

The Formation of Intercellular Junctions

While the mechanisms that build an electrical synapse are not well understood, critical clues to how the process might work are likely to be found in the known mechanisms that build other junction types such as adherens junctions, tight junctions, non-neuronal gap junctions, and chemical synapses. This process of junction formation requires: (1) selecting the junction site; (2) adhering to the cellular membranes in close apposition; (3) anchoring to the cytoskeleton; and (4) coordinating protein recruitment between the two cells to form a functional junction. Every junction type must create molecular solutions to these problems, and while each junction has its unique features, they share a common foundation (Fig. 2).

Figure 2 (next page). (A) Adherens junctions are the simplest junctions consisting of cell adhesion molecules (CAMs, blue) such as cadherins and nectins, and scaffolding proteins like Afadin (pink) combined with linker proteins (brown) such as catenins that connect cellular membranes to the actin cytoskeleton (purple). IC, Intracellular; EC, Extracellular. (B) Tight junctions use different CAMs (blue) including claudins and occludins to bring the neighboring cell membranes tightly together. These CAMs connect to the actin cytoskeleton (purple) *via* several scaffolding molecules (pink) including ZO proteins. (C) Non-neuronal gap junctions use Connexin proteins arranged in hexameric hemichannels (green) to intercellularly connect cells. Connexins also use scaffolding proteins (pink) including ZO proteins to link to other signaling molecules and the actin cytoskeleton (purple). (D) Chemical synapses, such as the glutamatergic excitatory chemical synapse represented here, have a vast assortment of proteins composing their structure including a variety of CAMs (blue), scaffolding molecules such as PSD95 (pink), neurotransmitters and synaptic vesicles (SV) and associated proteins (yellow and orange), neurotransmitter receptors and calcium channels (green), cytoskeletal adaptor proteins and other signaling molecules (brown), etc. PRE, Presynapse; POST, Postsynapse. (E) Electrical synapses are neuronal gap junction channels and use Connexins (green) to directly interconnect two neurons. Electrical synapses are often thought of as molecularly symmetric, but they can have asymmetric protein localization, as depicted here. At asymmetric electrical synapses, two postsynaptic proteins, ZO1 (pink) and Ca2+/calmodulin-dependent protein kinase II (CAMKII, brown) are observed to directly interact with Connexin C-terminal tails in the postsynapse to provide scaffolding and kinase activity. Due to the cell-biological specificity of electrical synapse formation within the complexity of neuronal morphology, and given their sophisticated functions in fast interneuronal communication, we expect that a large assortment of unknown proteins (gray) exists to manage electrical synapse formation and function. See the text for details. Republished with permission of Rockefeller University Press, from Brightman and Reese (1969); permission conveyed through Copyright Clearance Center, Inc.



In both neuronal and non-neuronal tissues, adherens, tight, and gap junctions exist to link cells to one another. Adherens junctions essentially take on the role of molecular glue between cells (Fig. 2A). These structures mediate cell-cell adhesion *via* the extracellular binding of cell adhesion molecules (CAMs), which include transmembrane cadherins and nectins (Troyanovsky, 2014). Intracellularly, CAMs anchor the cell membrane to actin *via* cytoskeleton-interacting linkers and scaffolding proteins such as catenins and afadin (Indra et al., 2013). By contrast, tight junctions bind cells to one another to create a seal that generates a mesh-like barrier with small pores between tissues. These junctions largely use the claudin CAM family as their transcellular connector and link to intracellular scaffolding proteins such as ZO proteins

(Figure 2B; Zihni et al., 2016). Unlike adherens and tight junctions, gap junctions create a physical intercellular channel connecting the two cell cytoplasms and making a direct passage for ions and other small molecules to pass from cell to cell. Gap junctions are created by coupled hemichannels contributed by each cell, with each hemichannel, in vertebrates, being comprised of a hexamer of Connexin proteins (Fig. 1C, 2C). Invertebrates accomplish the same task by using an evolutionarily distinct class of proteins called Innexins to form gap junctions (reviewed in Phelan, 2005; Güiza et al., 2018). Much like the CAMs at adherens and tight junctions, Connexins are intracellularly connected to scaffolding and cytoskeletal linkage proteins including ZO proteins and EB1 (Li et al., 2004; Epifantseva and Shaw, 2018). Thus, while there is some molecular overlap, each junction's unique morphology and function requires specialized membrane proteins, and fundamentally each must have a form of CAM, a scaffold, and an anchor to the cytoskeleton. How does this change within a neuronal environment?

Neurons use their special intercellular junctions to support the fast communication needs of neural network function. Moreover, the cell biological demands of their complex and diverse morphology (far-reaching axons and dendrites) require a carefully orchestrated protein delivery and control system (Tahirovic and Bradke, 2009). In particular, neuronal cells have two specialized junctions to manage fast information flow: chemical and electrical synapses. Chemical synapses (Fig. 2D) are fundamentally asymmetric structures, with the presynaptic side, the so-called active zone, specialized for fast synaptic vesicle release in response to neuronal action potentials (Südhof, 2012). Synaptic vesicle exocytosis at the active zone releases neurotransmitters into the synaptic cleft between the neurons to activate receptors on the postsynaptic cell. The postsynapse also termed the postsynaptic density, is specialized to manage the localization, organization, and function of neurotransmitter receptors to control communication (Frank and Grant, 2017). As with their non-neuronal junction counterparts, common mechanistic themes control the formation of all chemical synapses. Synaptic CAMs are thought to initiate synaptogenesis and offer trans-synaptic structural support; intracellular synaptic scaffolding molecules organize and stabilize both the pre and postsynaptic compartments; and adaptor proteins link to the cytoskeleton to manage trafficking,

anchoring, and later plasticity. Proteomic work on pre- and postsynaptic chemical synapses have revealed hundreds and thousands of proteins, respectively, in each compartment (Collins et al., 2006; Bayés and Grant, 2009; Ryan and Grant, 2009; Dieterich and Kreutz, 2016). While there is great protein diversity in these connections, each of the molecular aspects of building a chemical synapse relates to the fundamental themes of adhesion, scaffolding, and cytoskeletal anchoring, and these are critical to the structure, function, and plasticity of these connections.

While we know relatively little about the molecular mechanisms that regulate electrical synapses (Figure 2E), their observed functional diversity and plasticity suggests complex cell biological rules must control their formation and function, presumably using similar mechanisms as the other junction types. The notion of electrical synapse complexity is supported by several observations. First, we know that these neuronal gap junctions appear throughout the nervous system, from sensory neurons to central processing circuits to motor outputs (Galarreta and Hestrin, 2001; Connors and Long, 2004; Nagy et al., 2018). Besides, circuits build these connections in development and then refine them to form the final set of electrical synapses used in adulthood (Rash et al., 2000; Galarreta and Hestrin, 2002; Pereda, 2014). Thus, there must exist critical gene regulatory networks controlling when and where electrical synapse genes are expressed. Second, electron microscopy shows that the cell biological construction of electrical synapses is varied, and these structures can form between all neuronal compartments: there are axo-dendritic, somato-somatic, axo-axonic, and dendro-dendritic electrical synapses (Kosaka and Hama, 1985; Hamzei-Sichani et al., 2007; Nagy et al., 2018). These varied configurations suggest molecular specificity mechanisms to ensure electrical synapses are made in the right places and at the right times. Finally, electrical synapses are found in multiple morphological arrangements, such as in dense plaques, lacey plaques, wide ribbons, and thin strings (Nagy et al., 2018), suggesting that individual synapses are differentially regulated to achieve their unique functional needs. Here, we will explore the cell biological and molecular mechanisms which likely exist to manage each of these processes, beginning with gene expression control, then how gap junction proteins arrive at the synapse, followed by an analysis of electrical synapse organization, then by addressing how an electrical synapse site may be specified, and

finally by exploring how electrical synapses may contribute to disease. Our goal is to highlight critical areas of unexplored biology with the hope that this spurs efforts to identify the molecules and mechanisms that build, maintain, and allow for the modification of the electrical synapse.

Expression and Localization of Gap Junction Forming Genes

To make electrical synapses, neurons must express genes that support gap junction formation. In chordates, gap junctions are created by Connexins while in non-chordate animals Innexins make the channels (Slivko-Koltchik et al., 2019). While chordates retain Innexin genes, called Pannexins in these genomes, these proteins only make hemichannels and do not form intercellular junctions (Abascal and Zardoya, 2013). Despite evolution devising two molecular solutions to forming gap junctions, Connexin and Innexin structure and function are strikingly conserved (Goodenough and Paul, 2009; Pereda and Macagno, 2017). All animal genomes contain large numbers of gap junction forming genes, each expressed in cell-type-specific patterns and encoding proteins that facilitate unique functions. Therefore, to understand the electrical synapses of the nervous system, it is critical to examine the molecular complexities of the gap junctions. In *C. elegans*, 17 of the 25 Innexin genes are neuronally expressed, and they display highly complex and overlapping patterns that suggest incredible electrical synapse molecular complexity (Bhattacharya et al., 2019). Analogously, vertebrate genomes encode many Connexins; for example, zebrafish contain ~40 unique genes (Watanabe, 2017). Most Connexin genes are not expressed within neurons, such as the gene *gap junction a1 (gja1)* encoding the Connexin43 (Cx43) protein, which is expressed in non-neuronal tissue including epithelia and glia (Janssen-Bienhold et al., 1998; Güldenagel et al., 2000; Misu et al., 2016). A subset of Connexins are expressed in neurons, though each gene has a unique expression profile within the nervous system (Li et al., 2009; Rash et al., 2013; Klaassen et al., 2016; Song et al., 2016; Miller et al., 2017). For example, the *gjd2*/Cx36 family of genes are the most broadly expressed neuronal Connexins, found in neurons from the forebrain to the spinal cord within zebrafish and mouse brains (Condorelli et al., 1998; Connors and Long, 2004; Li et al., 2009; Söhl et al., 2010; Miller et al., 2017). By contrast, the mammalian *gja10*/Cx57

gene and its homologs in zebrafish are expressed exclusively in retinal horizontal cells (Söhl et al., 2010; Klaassen et al., 2016; Greb et al., 2018). Thus, while a complete accounting of vertebrate Connexin expression in the nervous system has not yet been achieved, it is clear that regulated expression contributes to the specificity of the electrical connectome.

In addition to gene regulatory mechanisms contributing to electrical synapse specificity, there are complexities as to whether two different Connexins can form a gap junction. For example, Cx43 expressed within glia cannot form gap junctions with neuronally expressed Cx36 (Rash et al., 2001; Koval et al., 2014). By contrast, many Connexin types can interact with one another, either within a hemichannel or between apposed cells. Given that many neurons express multiple Connexin proteins, there is the potential for a variety of Connexin arrangements within neuronal gap junctions (O'Brien et al., 2004; Rash et al., 2013; Palacios-Prado et al., 2014; Miller et al., 2017). These rules of engagement are certainly important for creating specific connectivity, yet we still lack the complete set of compatibility guidelines between the large family of Connexins. The spatial and temporal control of Connexin expression, coupled with the rules of Connexin engagement, provide both specificity and opportunities for complexity in the formation of electrical synapses. Future work is required to elucidate the complete molecular map of electrical synapse gene expression and protein usage in a complex vertebrate brain such as zebrafish.

While Connexin incompatibilities and expression are important for specificity, it is also clear that neurons are selective in where they form electrical synapses. An intriguing example of this is found within the mouse retina where the rod and cone photoreceptors express Cx36 and make electrical synapses with one another (Deans et al., 2002; Li et al., 2014; Asteriti et al., 2017). The photoreceptors also make chemical synapses with bipolar neurons, which themselves are coupled to other retinal neurons by Cx36-mediated electrical synapses (Deans et al., 2002; Trenholm and Awatramani, 2019). However, the photoreceptors do not make electrical synapses with bipolar neurons, despite their ability to form chemical synapses with one another and their mutual expression of Cx36. How can this be? The answer must arise from cell biological mechanisms that specify where the Connexins travel within the cell to form gap

junctions. Yet we know little about the trafficking mechanisms of Connexins within neurons.

Trafficking of Connexins within Neuronal Compartments

Most of our understanding of Connexin trafficking comes from studies of Cx43-based gap junctions (reviewed in Epifantseva and Shaw, 2018). In essence, Cx43 hemichannels are packaged into vesicles, travel along microtubules to an adherens junction situated near an established gap junction plaque, and are deposited into the membrane where they then migrate to and are incorporated into the plaque. However, in considering how electrical synapses are built, neurons offer additional trafficking challenges given their distinct cellular compartments. In most vertebrate neurons, axons are far-reaching processes that control information transmission at the presynapse, while dendrites are highly branched processes that typically stay relatively near the cell soma and manage information reception at the postsynapse. Axons and dendrites use analogous yet distinct processes to manage specific protein trafficking to their pre- and postsynaptic contact points. While chemical synapse contacts are necessarily asymmetric, electrical synapses can be either symmetric or asymmetric, and the flow of information at the electrical synapse can be bi-directional or biased (rectified; Phelan et al., 2008; Rash et al., 2013; Miller et al., 2017; Bhattacharya et al., 2019). In this review article, we will often refer to presynaptic (axonal) and postsynaptic (dendritic) electrical synapse components, and we do so only concerning the polarized neuronal compartments in which each side of the synapse resides. Given that electrical synapses occur on dendrites, cell bodies, and axons, and that axons and dendrites use different methods to traffic proteins, the trafficking of Connexins and other electrical synapse components within neurons must be controlled to build the appropriate electrical connections.

A striking example of the molecular organization of Connexins within distinct neuronal compartments was recently revealed using the power of zebrafish genetics. In zebrafish Mauthner neurons, two Connexins, Cx34.1 and Cx35.5, both homologous to mammalian Cx36, are necessary for electrical synapse formation (Miller et al., 2017). Surprisingly, Cx34.1 is specifically required in the postsynapse while Cx35.5 is

exclusively required in the presynapse, but the mechanisms guiding compartment-specific Connexin localization are unknown. This asymmetric compartmentalization of Connexins suggests that molecular rules must exist to guide specific Connexin types to particular sub-neuronal regions. Connexin proteins are four-pass transmembrane domain proteins with N and C-termini located intracellularly (Fig. 1D). Postsynaptic Cx34.1 and presynaptic Cx35.5 are ~90% amino acid identical, yet they have tantalizing differences in their intracellular loops and C-terminal tails which must, in some as yet undiscovered way, support their separate requirement in dendrites and axons. If we look to the chemical synapse for clues, we find that the trafficking and stabilization of postsynaptic AMPA neurotransmitter receptor subtypes are regulated through interactions between its C-terminal domain and intracellular scaffolding proteins, which connects them to the cytoskeleton and other signaling molecules (reviewed in Anggono and Huganir, 2012). But how do neurons target Connexins to these different neuronal compartments?

To traffic along axons and dendrites, Connexins first need to be packaged into vesicles which sort them into neuronal compartments according to the proteins on the vesicle surface. Identifying the types of vesicles in which Connexins transit would help us to understand their trafficking pathway, but these vesicles are yet to be identified. The vesicles must next engage with the intrinsic neuronal polarity mechanisms that define dendrites and axons, particularly the motor proteins that direct traffic along microtubules to these specific regions. These compartmental motors are distinctly organized: guidance to the presynapse along the axon requires kinesin motor proteins, and guidance to the postsynapse along the dendrite requires tethering to both kinesins and dyneins, with short-range, synaptic delivery in each compartment guided by actin-trafficked myosin motor proteins (for a detailed analysis of axon and dendrite polarity differences see Rolls and Jegla, 2015). Both tubulin (Brown et al., 2019) and actin (Wang, 2015) are required for proper trafficking of Cx36 to the membrane. Yet we still do not know the types of motor proteins Connexins or other electrical synapse components use to direct electrical synapse protein trafficking. However, recently some clues have started to point the field in the right direction.

Connexins likely rely on adaptor proteins to regulate their transport to the synapse. In a forward genetic screen using zebrafish, the epilepsy- and autism-associated gene Neurobeachin was identified as necessary for both electrical and chemical synapse formation (Iossifov et al., 2014; Miller et al., 2015; Mulhern et al., 2018). Neurobeachin is localized on vesicles which are found at the trans side of the Golgi, along dendrites, and also at chemical postsynapses (Wang et al., 2000; Miller et al., 2015). Its localization at electrical synapses is currently unknown. Past studies show Neurobeachin regulates membrane protein trafficking of chemical synapse scaffolds including PSD95 and SAP102 which in turn control the trafficking of neurotransmitter receptors (Medrihan et al., 2009; Niesmann et al., 2011; Nair et al., 2013; Farzana et al., 2016; Gromova et al., 2018). In zebrafish Mauthner neurons, Neurobeachin loss results in the failure of Connexin and electrical synapse scaffold ZO1 localization. Intriguingly, Neurobeachin is both necessary and sufficient postsynaptically for electrical synapse formation in this circuit (Miller et al., 2015). This supports a model wherein Neurobeachin controls the polarized trafficking of electrical components within the postsynaptic dendrite, although the molecular mechanism remains unknown. It is attractive to speculate that perhaps Neurobeachin acts to define dendritically targeted vesicles carrying electrical synapse cargo and that it may bridge them to the motor proteins required for postsynaptic delivery. Future experiments are required to identify how Neurobeachin functions in the dendrite to control synapse formation. The coordination of electrical and chemical synapses through a master synapse regulator such as Neurobeachin has critical implications for understanding the etiology of neurodevelopmental disorders (further discussed at the end of this review).

Once arriving at the synapse, Connexin vesicles must undergo exocytosis to become inserted into the membrane, allowing them to find their partner hemichannels in the neighboring neuron. Chemical synapses use v-SNAREs, present on pre and postsynaptic vesicles, to bind t-SNAREs on the neuronal membrane and fuse the vesicles at the synapse. Work in goldfish Mauthner neurons examined the effect of SNAP-25 peptides, which block the formation of SNARE-complexes, on the mixed electrical-chemical synapses of the Mauthner club endings (Flores et al., 2012). Mixed electrical-chemical synapses at single synaptic termini represent another fascinating synaptic

organization, and each component appears to be separately organized (Pereda, 2014; Nagy et al., 2019). Intra-dendritic application of these SNAP-25 peptides reduced both the electrical and the glutamatergic component of synaptic transmission suggesting the SNARE complex may function in Connexin insertion at the membrane (Flores et al., 2012). If the SNARE complex functions to fuse Connexin vesicles, there must be v-SNARE proteins within Connexin vesicles. But again, the composition of Connexin-containing vesicles and its protein constituents remain unknown. Insight into the molecular control of Connexin vesicle trafficking and membrane insertion in neurons will be critical to understanding electrical synapse formation and plasticity.

Further insights into the cell biological framework of electrical synapses will require an identification of the type of vesicles that contain Connexins; the motor, adaptor, and vesicle fusion proteins required for their transport and membrane fusion; and to determine if these features change between electrical synapse formation and plasticity. The elucidation of the cell biological pathways regulating electrical synapse protein trafficking will reveal whether they are the same or distinct from those of chemical synapses. The fact that electrical and chemical synapses have known distinct protein constituents suggests that at least some components will be unique, but the involvement of both Neurobeachin and SNAP-25 suggests some molecular overlap is also present. Besides, several trafficking conundrums remain. If Neurobeachin manages the postsynaptic trafficking of Connexins, what guides Connexin to the axon and the presynapse? And, in mammals, given that Cx36 is used within both the axon and the dendrite, how does a neuron resolve specific trafficking to these compartments? One possibility is that Connexin trafficking depends upon posttranslational modifications to the protein, such as phosphorylation (Li et al., 2009, 2013), to direct its localization. Or instead, Neurobeachin and other adaptor proteins may bind a scaffold protein which traffics with Connexin, as is observed with chemical synapse components (Tao-Cheng, 2007; Vukoja et al., 2018). Thus, cell type-specific expression of these scaffolds and adaptors could result in different trafficking patterns and thus different cell biological construction of electrical synapses. This leads us to our next question: how do electrical synapse scaffolds control electrical synapse development?

Organizing the Growing Electrical Synapse

To fully appreciate electrical synapse cell biology, we must understand that each electrical synapse is composed of plaques of tens to thousands of gap junction channels (Flores et al., 2012; Rash et al., 2012, 2013, 2015; Yao et al., 2014). These plaques of gap junction channels can take on many different conformations such as wide or thin ribbons and large circular regions of channels, either densely collected or with lace-like holes (Nagy et al., 2018). Connexins arrive at the synapse as hemichannels that are inserted at the boundaries of existing gap junction plaques where they then find a partner hemichannel in the adjoining neuron. Over time, the channels migrate towards the center of the plaque where they are endocytosed and sent to the lysosome for degradation (Lauf et al., 2002; Flores et al., 2012; Wang et al., 2015). The half-life of Cx36 is estimated to be between 1 and 3 h *in vivo*, so to maintain the electrical synapse, Cx36 must continuously be made and trafficked to the correct location (Flores et al., 2012; Wang et al., 2015). The known organizational principles of the plaque, and the turnover demand of Connexins, requires complex and ongoing molecular machinery to ensure appropriate development and homeostasis. But what ensures that the components of the electrical synapse, including Connexins, unite at the same place over time?

The gene *tjp1* encodes the ZO1 protein, a membrane associated guanylate kinase (MAGUK) historically known for its necessity at tight junctions (Umeda et al., 2006) and epithelial gap junctions (Singh et al., 2005; Bao et al., 2019), and first identified at electrical synapses in the mouse brain (Li et al., 2004; Penes et al., 2005). Recent work in zebrafish shows that ZO1 is required for electrical synapse formation (Marsh et al., 2017) as larval fish mutant for the ZO1 homolog *tjp1b* lack Connexin localization resulting in functional deficits at electrical synapses. This suggests Tjp1b/ZO1 is required to either recruit, traffic, or stabilize Connexins at electrical synapses. Strikingly, the broad class of MAGUK scaffold proteins are well-known for their ability to aggregate protein components at other well-studied cell-cell junctions (see Fig. 2B–E, MAGUKs shown in pink). For example, PSD95, SAP102, and PSD93 are all postsynaptic MAGUK proteins that localize at glutamatergic chemical synapses, make up a majority of proteins in the postsynaptic density, and interact either directly or

indirectly with glutamatergic neurotransmitter receptors. Simultaneous knock-down of these three scaffolds results in smaller postsynaptic densities and a substantial reduction in chemical synapse transmission (Chen et al., 2015). These findings support MAGUKs, including ZO1, as master organizers of intercellular junctions. The unique features that facilitate their shared function at different cell-cell adhesions are exhaustively reviewed elsewhere (e.g., Zhu et al., 2016; Ye et al., 2018), but we will highlight several key characteristics that inform our understanding of ZO1 at the electrical synapse.

First, MAGUK proteins contain one or more PDZ (PSD95, Dlg1, and ZO1) domains. These domains interact with short ligand sequences, called PDZ binding motifs (PBMs), usually found at the C-terminus of the interacting protein. At cell-cell junctions, MAGUK PDZ domains bring together the C-termini of transmembrane (or auxiliary) proteins to create a carefully organized hub of molecular interactions (reviewed in Lee and Zheng, 2010). Although all PDZs share a canonical structure, amino acid differences in the binding surface of the PDZ and PBM confer interaction specificity (Giallourakis et al., 2006; Liu et al., 2019). Additionally, these specific interactions can be regulated by posttranslational modifications to either the PDZ or the ligand motif. At the electrical synapse, Cx36 and its teleost homologs all contain a C-terminal SAYV motif that interacts directly with the first PDZ domain of ZO1 (Li et al., 2004; Flores et al., 2008). It has, therefore, been proposed that electrical synapse formation and function requires a ZO1-PDZ1/Cx36- PBM interaction, but this has yet to be explicitly shown *in vivo*.

Second, in addition to transmembrane proteins, MAGUKs also interact with other scaffolds, regulatory proteins, signaling proteins, the cytoskeleton, and even in some cases the plasma membrane. This array of interactions allows MAGUKs to aggregate the pieces necessary to create, maintain, and regulate a functional junction. ZO1 is found in complex with numerous proteins found at the electrical synapse including neuronal Connexins (Li et al., 2004; Flores et al., 2008), CAMKII, which is responsible for some forms of electrical synapse plasticity (Alev et al., 2008; Flores et al., 2010; Li et al., 2012), and actin (Fanning et al., 2012). Thus, ZO1 appears poised to act as the central hub for electrical synapse protein organization and to act as a direct link to the

cytoskeleton, yet the details of how it achieves this molecular coordination remain unknown.

Finally, recent studies have shown that many MAGUK proteins are capable of phase separating, creating dynamic and selective non-membrane bound organelles. All MAGUKs include a PDZ-SH3-GUK (PSG) tandem set of domains that function in regulated oligomerization (Pan et al., 2011; Rademacher et al., 2019), thus creating highly concentrated nanodomains that can aggregate various proteins to a specific site within a cell. At chemical synapses, phase separation within the presynaptic active zone clusters synaptic vesicle fusion proteins while at the postsynaptic density phase separation concentrates neurotransmitter receptors (reviewed in Chen et al., 2020). Recent work has found that ZO1 is capable of phase separation facilitated by its PSG tandem, and loss of ZO1's phase separating capabilities in mammalian cell culture and the larval fish results in a loss of aggregation near the epithelial membrane and impairments in tight junction integrity (Beutel et al., 2019; Schwayer et al., 2019). Thus, it is attractive to propose a model of electrical synapse formation led by ZO1 phase separation which provides a local, specialized domain to capture Connexins and other molecular machinery through both direct and indirect interactions. This presents an exciting new avenue for future exploration.

Our knowledge of ZO1 and other MAGUKs at cell-cell junctions suggests a model in which ZO1 is oligomerized into nanodomains at the cell membrane destined to become Connexin plaques. As Connexins are rapidly turned over throughout the life of the electrical synapse, ZO1 stabilizes them, aggregates necessary regulatory proteins such as kinases, and links the structure to the cytoskeleton. Intriguingly, ZO1 has been shown to interact with numerous neuronally expressed Connexins, in addition to Cx36, suggesting that this mechanism may be common across all electrical synapses (reviewed in Hervé et al., 2012). The emerging evidence suggests ZO1 acts as a master organizer of electrical synapses once it is recruited to the site of the future electrical synapse. This, however, leads us to the question: what tells ZO1 where the electrical synapse should be?

Specifying When and Where Electrical Synapses are Created

Although it is possible that site specification initially occurs *via* extracellularly secreted signals, we know that synaptic initiation and maintenance requires cell adhesion molecules (CAMs). These membrane-spanning proteins have extracellular domains allowing for intercellular interactions with CAMs on an opposing cell. Additionally, they have intracellular domains that interact with the cytoskeleton, scaffolds, and other proteins that can trigger signaling cascades and the recruitment of other molecules. Thus, it is highly likely that neurons use CAMs to choose the right place and the right time to create an electrical synapse.

Could the Connexin proteins act as the CAM for electrical synaptogenesis? Connexins are indeed CAMs, and, in certain circumstances such as radial migration of neurons in the mouse cortex, the adhesive properties appear to be more important than the channel itself (Elias et al., 2007). So it is tempting to question if Connexins coordinate the recruitment of ZO1 and other required proteins to the electrical synapse. The gap junction channel as director of synapse formation appears to be the case in the leech, where the diversity of gap junction forming Innexin proteins drives the site-specific formation of electrical synapses (Baker and Macagno, 2014). However, in vertebrates, which use Connexins for their gap junctions, this may not be the case. In *Cx36* mutant mice that lack many neuronal gap junctions, electron microscopic analysis of the stereotyped dendro-dendritic electrical connections between olivary neurons found recognizable intercellular junctions still formed, but they lacked the classic electron-dense, gap junction morphology (De Zeeuw et al., 2003). A similar conclusion was found using immunohistochemistry at the MesV nucleus in *Cx36* null mice, where the stereotyped electrical synapse lacked neuronal Connexin staining, yet ZO1 was still localized to the putative electrical synaptic sites (Nagy and Lynn, 2018). Taken together, these results suggest that electrical synapses are specified by mechanisms other than Connexins, yet the nature of the signal remains unknown.

So what are the CAMs that specify electrical synapse sites? Vertebrate genomes contain thousands of genes that encode CAMs (Zhong et al., 2015), making it no small feat to identify the correct molecules that initiate electrical synapse site specification. Yet particular CAMs, such as the Nectins, may be the key as they play a critical role in

establishing initial cell-cell adhesions and are known for their instructive role in adherens junction and tight junction formation in epithelia. At these locations, they precede the cadherin-based or claudin-based adhesions that are recruited later to these sites. Nectins build up a macromolecular complex by interacting with Afadin, an intracellular scaffold that directly interfaces with the actin cytoskeleton and other important scaffolds, such as alpha-catenin and ZO1, required for adherens junction and tight junction formation respectively (Yamada et al., 2006; Ooshio et al., 2010). In neurons, the loss of Nectins results in altered axon targeting whereas loss of Afadin results in greatly decreased neuronal N-cadherin and β- and αN-catenin puncta along with extensive reductions in excitatory synapse density (Honda et al., 2006; Beaudoin et al., 2012). The effects on electrical synapses have not been assessed. The relationship between Nectins and Afadins is likely cell type-specific, but these results support that, much like at tight junctions, these complexes form initial adhesions that lay a foundation for cadherin recruitment to the synaptic site.

But are Nectins responsible for specifying the locations of electrical synapses? Cx36, ZO1, and Afadin, but not Nectin, colocalize at electrical synapses in the rat/mouse brain. Moreover, Cx36 co-immunoprecipitates with Afadin in both whole-brain and retinal homogenates (Li et al., 2012), most likely through direct interaction with ZO1. Adjacent to electrical synapses, Afadin is also present at adherens junctions where it colocalizes with Nectin and N-cadherin (Li et al., 2012; Nagy and Lynn, 2018). This suggests a potential model where initial Nectin/Afadin adherens junctions form between neurons before electrical, or chemical, synapse formation and they recruit in cadherins to maintain the synapse, however, this has not been explicitly tested. How specification proceeds to differentiate between these future structures to guide specific molecular complex formation or whether these are causally required for formation remains unclear.

Alternatively, electrical synapses may use different complements of CAMs in their formation and maintenance, and to potentiate their functional plasticity. Chemical synapses use a multitude of synaptic CAMs not only to specify separate synaptic types (e.g., excitatory and inhibitory) but also to solidify and modulate synapse connections between neurons over time (Jang et al., 2017; Rawson et al., 2017). Other CAMs, such as claudins, occludins, and N-cadherin, all are found to interact with Connexins in epithelia

alluding to their potential roles at the electrical synapse (reviewed in Hervé et al., 2012). However, attempting to elucidate the requirement of these CAMs *in vivo* is difficult due to the pleiotropic nature of these proteins and their use at many cellular junctions. So how can the electrical synapse CAMs be identified and studied? Zebrafish offer some advantages, particularly given the new methods in CRISPR-based reverse genetic screening (Shah et al., 2015), which provides a fast method for knocking out a large battery of potential CAMs to identify those that regulate electrical synapses. For the field, identifying the CAMs that specify the temporal and spatial electrical synapse dynamics is an essential hurdle that needs to be overcome to move forward in understanding the cell biology of the electrical synapse.

Discussion and Conclusions

Here we have explored several critical open questions surrounding the cell biology of the electrical synapse. Filling these gaps in knowledge will greatly impact our understanding of the development and homeostasis of electrical synapses and will provide new frontiers in regard to the etiology of neurological disorders.

Numerous human disorders are characterized by the loss of gap junction channels, and they span tissues including the skin, heart, joints, teeth, and immune system, to name just a few (Jongsma and Wilders, 2000; van Steensel, 2004; Kleopa and Scherer, 2006; Laird, 2006, 2010; Wong et al., 2017; Donahue et al., 2018). Indeed, the leading cause of deafness is due to the loss of Connexins expressed in the ear, which is currently, and extremely controversially, earmarked for a possible human CRISPR trial (Batissoco et al., 2018; Cyranoski, 2019). These pathologies seemingly emerge from the disruption of wide-ranging gap junction roles within cell proliferation and differentiation, morphogenesis, cell migration, growth control, and many other cell biological processes (McGonnell et al., 2001; Vinken et al., 2006; Kardami et al., 2007; Marins et al., 2009). If we turn our gaze to the nervous system, we find that in *Cx36* knockout mice there are brain-wide electrical synapse defects such as within the cerebellum where motor function is impaired, in the hippocampus where perturbed long-term potentiation and network oscillations impact learning and memory, in the cortex where cortical interneurons become desynchronized, and in both visual and

olfactory systems which are dysfunctional (Güldenagel et al., 2001; Frisch et al., 2005; Bissiere et al., 2011; Wang and Belousov, 2011; Zolnik and Connors, 2016; Pouille et al., 2017). Similar disruptions are mirrored in zebrafish, where elimination of *Cx36* homologs results in delayed responses to threatening stimuli and motor coordination defects (Miller et al., 2017). These behavioral defects in animal models lacking a broad class of electrical synapses are exactly what the field of neurodevelopment would expect for genes linked to disease phenotypes (Mas et al., 2004; Hempelmann et al., 2006; Solouki et al., 2010; Li et al., 2015; Kunceviciene et al., 2018). Namely, that many disorders of neurodevelopment result not in large effects with gross dysfunction, but instead are comprised of subtle molecular differences that slightly shift the functional outcomes. Indeed, many so-called synaptopathies are thought to affect synapse formation and perturb excitatory/inhibitory balances (Grant, 2012). We suggest that the perspective should be broadened to the electrical/excitatory/inhibitory balance, as disruptions to any of these components lead to subsequent abnormal circuit function which develops to have larger behavioral ramifications over time. Indeed, electrical synapse disruptions are proposed to contribute to the etiology of disorders such as autism (Welsh et al., 2005) and epilepsy (Cunningham et al., 2012). However, Connexin loss is not yet a well-appreciated contributor to such disorders. We think it is likely that the growing awareness and attention electrical synapses are receiving in neural circuit formation, function, and behavior will bring to light their links to a large set of neurodevelopmental disorders.

In this review, we have made the case that Connexins are not the full story in considering the form and function of the electrical synapse. Indeed, our work on Neurobeachin, which itself is linked with both autism and epilepsy in human patients, suggests that as we begin to understand the totality of electrical synapse formation, how these structures are related to disorders of neural function will become ever more apparent. Therefore, we fundamentally need to expand our understanding of the cell biological mechanisms that develop, maintain, and regulate electrical synapses. And we need to improve our knowledge of the mechanistic relationship between electrical and chemical synapse formation to clarify the contributions of each synapse type to development and adult neural circuit function. In conclusion, we predict that the

continuing studies of electrical synapse structure and function will provide a new framework for understanding fundamental mechanisms of brain structure and function as well as the etiology of the disease.

CHAPTER III

ZO1B IS REQUIRED FOR ELECTRICAL SYNAPSE FORMATION IN THE ZEBRAFISH HINDBRAIN

All figures and experiments in this chapter were performed, overseen, and/or analyzed by me. I performed the majority of the genetics, histology, and imaging with assistance from Audrey Marsh (Fig. 2, 4), Anne Martin (Fig. 6), and Elisa Trujillo (Fig. 6). I performed all software development and data analysis. I cloned the Connexin tails (Fig. 7), purified all interaction domains, and performed the binding assay with guidance from Jen Michel. Adam Miller identified and mapped *dis2* (Fig. 2, 3). Adam Miller and I conceived of the project and designed experiments. I created the figures and wrote the chapter.

Select data and methods are included in:
Lasseigne AM*, Echeverry F*, Ijaz S*, Michel JC*, Martin EA, Marsh AJ, Trujillo E, Marsden KC, Pereda AE, Miller AC (2021). Electrical synaptic transmission requires a postsynaptic scaffolding protein. *eLife*, 10:e66898 DOI: 10.7554/eLife.668.
* denotes co-first author

Introduction

Electrical and chemical synapses are specialized cellular adhesions that allow neurons to pass signals throughout a circuit. Uncovering the process by which cells correctly localize critical synaptic machinery has been at the forefront of neuroscience research for decades. In the case of chemical synapses, where presynaptic vesicles release neurotransmitters to influence postsynaptic receptors, we understand much about how these structures are organized and regulated. By contrast, the mechanisms by which electrical synapses, where gap junction channels physically connect the cytoplasm of two neurons, are formed remains largely unknown.

Electrical synapses are dynamic plaques of gap junction channels, with each channel being composed of two hexameric Connexin (Cx) hemichannels contributed by each adjoining cell (Goodenough & Paul, 2009). We know that these synapses come in many structural variants, occur between stereotyped synaptic partners, and are highly

dynamic (see Chapter II). Thus, they must require a level of organization that is robust enough to recruit and maintain Connexin plaques between precise partners but also flexible enough to allow for structural diversity and regulation. These properties of electrical synapses suggest that there must be underlying molecular support beyond the Connexin channels.

We know that chemical synapses are supported by a network of associated proteins that are largely organized by postsynaptic scaffolding molecules (Ye et al., 2018; Chen et al., 2015; Ackermann et al., 2015; Grant, 2019; Ye et al., 2018; Siddiqui and Craig, 2011). Scaffolds are specialized molecules containing multiple protein-protein binding domains to facilitate interactions between membrane-bound and intracellular proteins. All known cellular junctions require scaffolding proteins in some capacity to anchor membrane proteins to the molecules necessary for regulation and structural integrity (see Chapter II). Thus, we hypothesized that electrical synapses must also require a molecular scaffold.

To identify scaffolding proteins necessary for electrical synapse formation, we used the Club Ending synapses (CE) found on the lateral dendrites of zebrafish Mauthner cells (Bartelmez, 1933; Kimmel, 1982). The available genetic tools in zebrafish allow us to easily perturb the system and monitor changes in electrical synapse formation. Zebrafish have two Mauthner cells, one on each side of the body with dendrites and soma located in the hindbrain and an axon descending contralaterally down the length of the spinal cord. CE electrical synapses are formed from auditory afferent neurons contacting the Mauthner lateral dendrite in the zebrafish hindbrain. At 5 days post-fertilization (dpf), there are ~6-9 CEs per Mauthner cell, each around 2µm in diameter. The large, highly stereotyped nature of CEs provide a readout of electrical synapse formation and make them an ideal model to determine which molecules are necessary.

Here, we use forward and reverse genetics to identify Zonula occludens-1b (ZO1b; encoded by *tjp1b)*, a MAGUK scaffolding protein, to be required for robust Connexin localization to CEs. We also show that ZO1b localizes to CEs even in the absence of the neuronal Connexins but at much lower levels. This suggests a genetic hierarchy at CEs where ZO1b is necessary to recruit and stabilize Connexins at the synapses but where Connexins may also be stabilizing ZO1b. Furthermore, we show that

ZO1b is capable of a direct interaction with the neuronal Connexins and hypothesize that this biochemical interaction may be responsible for the hierarchical genetic relationship we identified between the channel proteins and the scaffold.

Results

The dis2 mutation disrupts *tjp1b* and electrical synapse formation

To identify scaffolding molecules required at the electrical synapse, we used Club Ending (CE) synapses in the larval zebrafish hindbrain as a model (Bartelmez, 1933; Kimmel, 1982). Zebrafish have two Mauthner cells, one on each side of the body with dendrites and soma located in the hindbrain and an axon descending contralaterally down the length of the spinal cord (Fig.1A). In the hindbrain, auditory afferents synapse onto the lateral dendrites of Mauthner where each creates an individual CE synapse (Fig. 1B). We examined electrical synapses in zebrafish at 5 days post-fertilization (dpf), when there are ~6-9 CEs per Mauthner cell. Each CE contains plaques of Connexin channels composed of asymmetric hemichannels. The presynaptic compartment within the auditory afferent uses Cx35.5 while the postsynaptic compartment within Mauthner uses Cx34.1 (Fig. 1C). These asymmetric Connexin hemichannels are interdependent i.e., loss of either Connexin inhibits the other from properly localizing to the synapse (Miller et al., 2017).

These robust, highly stereotyped synapses can be identified as large (~2µm wide at 5dpf) discs or crescents located around the lateral dendritic bifurcation of Mauthner. Both Cx35.5 and Cx34.1 are homologs of mammalian Cx36 and thus both are identified by anti-human-cx36 antibodies (Fig. 2A). To identify genes required for electrical synapse formation, we used N-ethyl- N-nitrosourea (ENU) to generate random genomic mutations and stained larval zebrafish with anti-human-Cx36 to screen for disruptions in electrical synapse formation. This forward genetic approach identified the *disconnect*2 (*dis2)* mutation. Animals heterozygous for this mutation showed decreased levels of Cx36 staining at CEs (Fig. 2B), and those homozygous for *dis2* showed an almost complete loss of Cx36 staining (Fig. 2C). This suggested that at least one of the

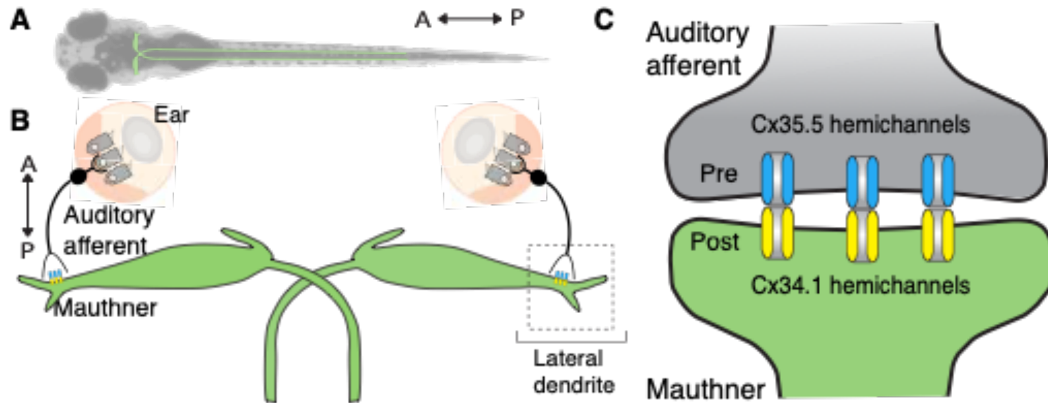


Figure 1. Simplified diagram of the Mauthner Club Ending (CE) electrical synaptic contacts
(A) Dorsal view of 5dpf zebrafish larvae (anterior to the left) with Mauthner cells depicted in green. (B) Diagram represents a dorsal view with anterior on the top. Boxed region indicates area of stereotypical CE synaptic contacts used for analysis. Presynaptic auditory afferents contact the postsynaptic Mauthner cell lateral dendrite in the hindbrain forming electrical synapses. (C) Model depicts asymmetric Connexin (Cx) organization of CE synapses. Electrical synapses are denoted as rectangles (B) or channels (C) depicting the two Connexin hemichannels (presynaptic Cx35.5 (cyan) and postsynaptic Cx34.1 (yellow)) that form the neuronal gap junction channels.

mutations present in the *dis2* animals is within a gene required for electrical synapse formation.

Before we could characterize the effects of *dis2* on Connexin localization, we needed to develop a method that allowed us to quantify the amount of staining seen at CE synapses. The image data gathered at CEs is in the form of multi-channel, three-dimensional stacks centered around the lateral dendritic bifurcation of the Mauthner cells (Fig. 2D). One channel contained information about the zf206Et transgene (labeling Mauthner; referred to as Mauthner:GFP or M/CoLo:GFP) with additional experimental channels containing the staining information we want to quantify (e.g. Cx36). Using Python, I developed an automated pipeline that took these stacks, isolated only CE staining, and calculated the integrated density of fluorescence for each experimental channel (Fig. 2E). To isolate synaptic staining, raw images were passed through a 3^3 median filter to reduce noise, thresholded (consistent within each experiment) to reduce non-synaptic staining, and Mauthner:GFP transgene labeling was used to remove any staining outside of the dendrite. I then calculated the integrated density for each Mauthner

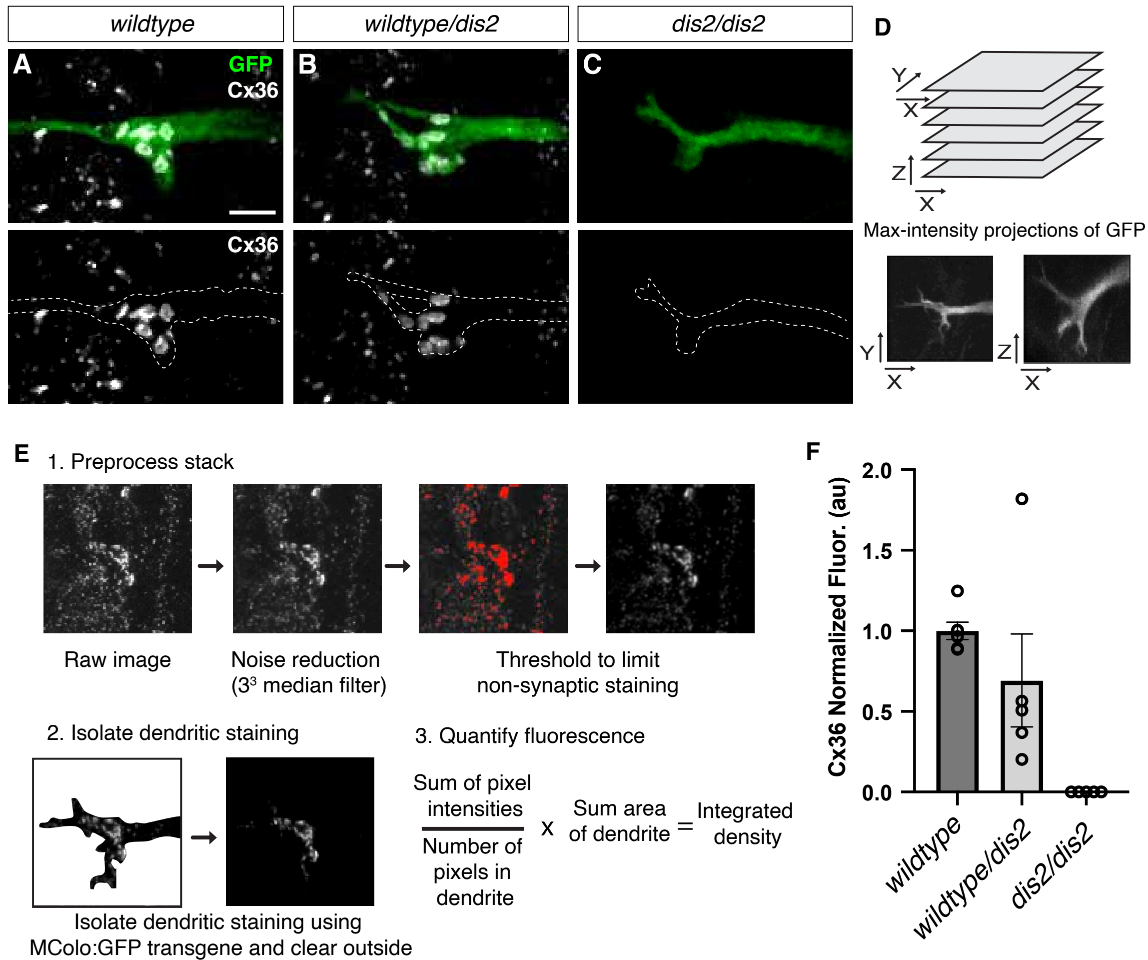


Figure 2. ***dis2*** **disrupts electrical synapse formation at CEs**
(A-C) Confocal images of Mauthner lateral dendrite and stereotypical CE electrical synaptic contacts in 5-day-post-fertilization, *zf206Et* zebrafish larvae from *wildtype* (A), *dis2* heterozygotes (B), and *dis2* homozygotes (C). Animals are stained with anti-GFP (green, white dotted outline) and anti-human-Cx36 (grey). Scale bar = 5 µm in all images. (D-E) To quantify the effect of *dis2* on Connexin staining, we developed a high-throughput, automated pipeline to process and quantify fluorescence staining at CEs. (F) Quantitation of Cx36 fluorescence at CEs in *wildtype* (n=6), *dis2* heterozygotes (n=5), and *dis2* homozygotes (n=5). The height of the bar represents the mean of the sampled data normalized to the *wildtype* average for a given experiment and circles represent the normalized value of each individual animal. Error bars are ± SEM.

cell, averaged within the animal, and normalized to *wildtype*. When we passed *wildtype*, *dis2* heterozygotes, and *dis2* homozygotes through this pipeline, our quantification supported our qualitative assessment that a mutation in *dis2* mutant animals disrupts electrical synapse formation as determined by Cx36 staining (Fig. 2F).

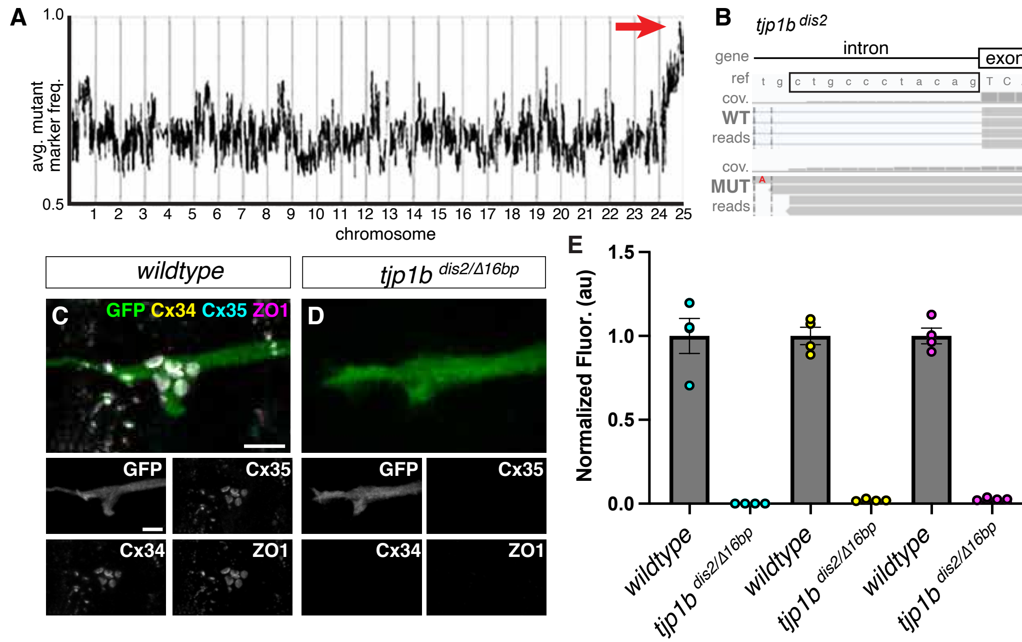


Figure 3. *dis2* is a mutation in the gene *tjp1b*
(A) Genome wide RNA-seq-based mapping data. The average frequency of mutant markers (black marks) is plotted against genomic position. A single region on chromosome 25 (chr25) emerges with an allele frequency near 1.0 indicating linkage to the *dis2* mutation (red arrow). Each chromosome is separated by vertical lines and labeled at the bottom. (B) Mutant reads from the RNA-seq mapping data within the *tjp1b* gene (encoding ZO1b) are shown aligned to the reference genome identifying a single base pair change causing the inclusion of intronic sequence. Wildtype reference (ref) genome nucleotides and encoded amino acids (aa) are noted. Aligned mutant (MUT) reads are shown as grey boxes; colored letters highlight differences from reference. (C-E) Electrical synapses are lost in trans-heterozygous animals carrying *dis2* and a 16bp deletion in *tjp1b* (Welch's t-test, $p<.05$). Images are of Mauthner lateral dendrites from *wildtype* (C) and *tjp1b dis2/Δ16bp* (D) animals stained with anti-GFP, anti-zebrafish-Cx35.5, anti-zebrafish-Cx34.1, and anti-mouse-ZO1. Adjacent panels show individual channels. Images were passed through a median filter. (I) Quantitation of Cx35.5 (cyan), Cx34.1 (yellow), and ZO1 (magenta) at CEs in specified genotypes (*wildtype* n= 4, *dis2/Δ16bp* n=4). The height of the bar represents the mean of the sampled data normalized to the wildtype average for a given experiment, and circles represent the normalized value of each individual animal. Error bars are ± SEM.

Next, we needed to find the causative mutation in the *dis2* animals that resulted in electrical synapse loss. We used an RNA sequencing (RNA-seq)-based approach that identifies shared regions of genomic homozygosity in a pool of mutant animals (Miller et al., 2013). The sequences were aligned to the genome and single nucleotide polymorphisms (SNPs) were identified in the wildtype pool to serve as mapping markers. The SNP frequency was assessed in the mutant pool, identifying a region of

homozygosity on chromosome 25 in *dis2* mutants (Fig. 3A). Within this region, we used the RNA-seq data to look for potentially deleterious mutations and found a single base pair change in the intronic region of *tjp1b* that resulted in the inclusion of intronic sequence in RNA reads (Fig. 3B). *tjp1b* encodes the protein ZO1b, a homolog of mammalian ZO1. To definitively determine whether the *tjp1b* mutation was causative to the phenotype, we used CRISPR mutagenesis to create a 16bp deletion in *tjp1b* resulting in a frame-shift mutation early in the coding sequence. I performed a complementation test, creating trans-heterozygous animals for *dis2* and *tjp1bΔ16bp.* These alleles did not complement, and both Cx34.1 and Cx35.5 were lost at CEs in trans-heterozygotes (Fig. 3D-E). Therefore, we concluded that mutations in *tjp1b* disrupt electrical synapse formation. We also wanted to determine where the ZO1b protein was acting. When we stained *wildtype* animals with an antibody against mammalian ZO1, we saw colocalization of ZO1 with Cx34.1 and Cx35.5 at CEs (Fig. 3C). In trans-heterozygotes, however, ZO1 staining was lost at the electrical synapses suggesting that what we observed at the synapse was the gene product of *tjp1b* (Fig 3D-E).

ZO1b localizes to CEs and is required for robust Connexin localization

Now that we had identified *tjp1b* as being required for electrical synapse formation, I wanted to characterize the deletion mutant in more detail. In *tjp1b*$^{\Delta 16bp/\Delta 16bp}$ animals, I saw a loss of the neuronal Connexins at the CEs, phenocopying what was observed in *dis2* mutants (Fig. 4C, D). We also saw a loss of ZO1 staining at CEs in mutant animals, as we saw in the trans-heterozygotic animals, further supporting that the protein detected at CEs is ZO1b, the gene product of *tjp1b*. Interestingly, when I looked at *tjp1b*$^{+/\Delta 16bp}$ animals, we saw a significant decrease in the levels of Cx34.1 staining at CEs but not in Cx35.5 (Fig. 4C, D). I also saw a corresponding decrease in ZO1 staining at the synapse. We also created a deletion mutation in *tjp1a*, the paralog of *tjp1b*, to see whether it played a similar role in synapse formation (Fig.4E). In homozygous *tjp1a* mutant animals, however, Cx34.1, Cx35.5, and ZO1 staining appeared normal at CEs (Fig. 4F). Taken together, the results show that ZO1b localizes to electrical synapses and is required for electrical synapse formation.

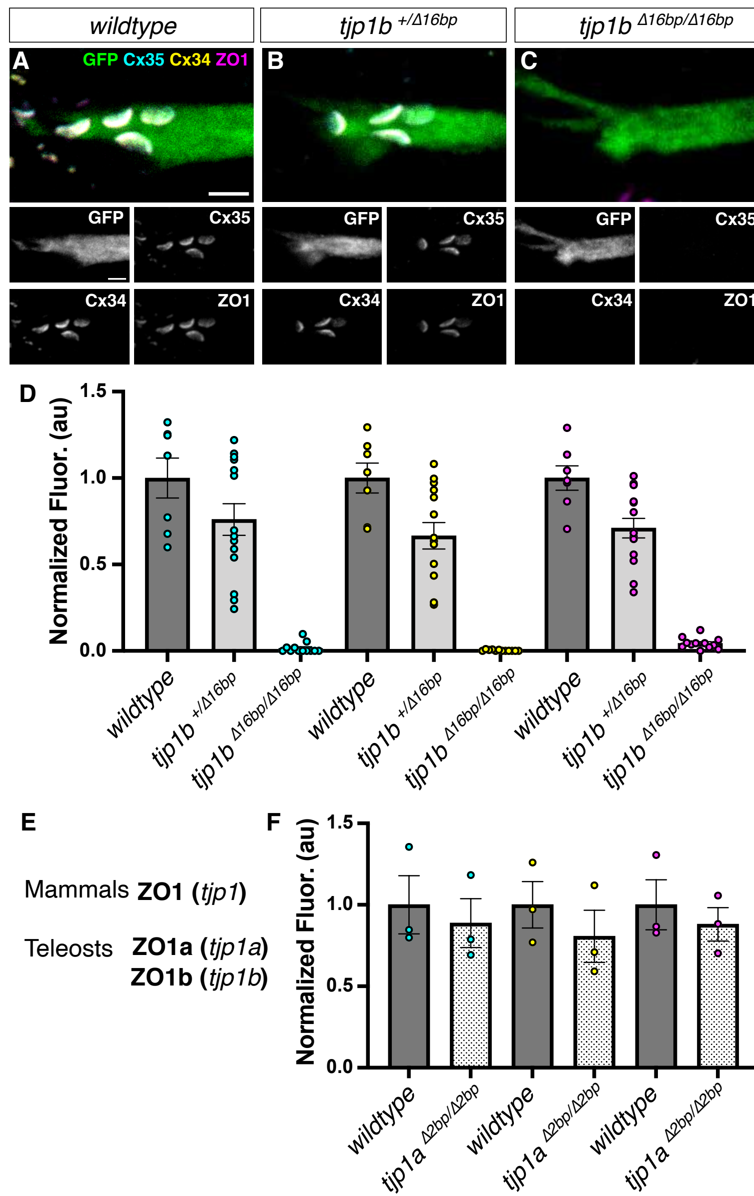


Figure 4. ZO1b localizes to CEs and is required for electrical synapses formation (A-C) Confocal images of Mauthner lateral dendrite and stereotypical CE electrical synaptic contacts in 5-day-post-fertilization, *zf206Et* zebrafish larvae from *wildtype* (A), *tjp1b* $^{+/\Delta 16bp}$ (B), and *tjp1b* $^{\Delta 16bp/\Delta 16bp}$ (C) animals stained with anti-GFP, anti-zebrafish-Cx35.5, anti-zebrafish-Cx34.1, and anti-mouse-ZO1. Adjacent panels show individual channels. Scale bar = 2 μm in all images. (D, F) Quantitation of Cx35.5 (cyan), Cx34.1 (yellow), and ZO1 (magenta) at CEs in specified genotypes. (D) Connexin and ZO1 staining are lost in *tjp1b* homozygous mutants, and Cx34.1 and ZO1 staining is reduced in heterozygous animals (*wt* n=7, *tjp1b+/-* n=14, *tjp1b-/-* n=12; ANOVA with multiple comparisons, p<.05). (E) *tjp1a* and *tjp1b* are homologs of mammalian *tjp1*. (F) Connexin and ZO1 staining are not impacted by the loss of *tjp1a* (*wt* n=3, *tjp1a-/-* n=3). The height of the bar represents the mean of the sampled data normalized to the wildtype average for a given experiment, and circles represent the normalized value of each individual animal. Error bars are ± SEM.

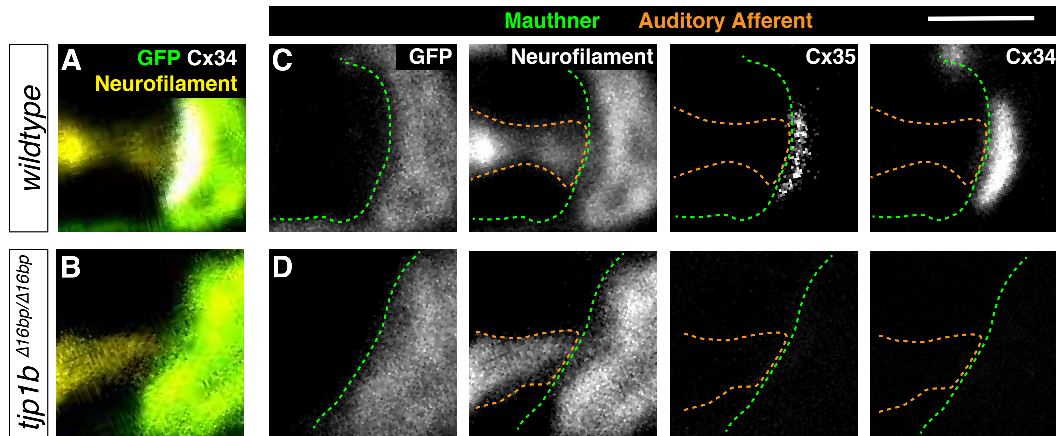


Figure 5. ZO1b is required for Connexin channel localization to CE synapses Auditory afferent contacts onto Mauthner are maintained in *tjp1b* mutant animals. Confocal images of *wildtype* (A,C) and *tjp1b* $^{\Delta 16bp/\Delta 16bp}$ (B,D) animals stained with anti-GFP, anti-zebrafish-Cx35 (not shown in merge), anti-zebrafish-Cx34, and anti-RMO44 (labeling neurofilament). (C,D) Panels show individual channels. Green dotted line depicts Mauthner cell (labeled by GFP) and orange dotted line indicates auditory afferents (labeled by neurofilament marker). Scale bar = 2 µm.

One possibility for the loss of staining at the CEs is that the auditory afferents are no longer present or contacting the Mauthner dendrite. To test this, we stained *tjp1b*$^{\Delta 16bp/\Delta 16bp}$ animals with a neurofilament marker to label the auditory afferents in addition to GFP and the neuronal Connexins. Mauthner was co-labeled by both GFP and the neurofilament marker, while auditory afferents were only labeled by the neurofilament marker. When I zoomed in on a single CE in a *wildtype* animal, I could see the auditory afferent meeting the Mauthner dendrite at the site of the Cx34.1 and Cx35.5 staining (Fig. 5A,C). In *tjp1b*$^{\Delta 16bp/\Delta 16bp}$ animals, I could also see the auditory afferents coming into contact with Mauthner even though Connexin staining was lost (Fig. 5B,D). Thus, neuronal morphology is maintained in animals lacking *tjp1b,* but Connexin localization to contact sites is lost.

<u>ZO1b localizes to the electrical synapse independent of Connexins</u>

Given that Connexin localization was dependent on ZO1b, we sought to determine if the converse was true – did ZO1 localization require Connexins? Using previously generated null mutations in *cx35.5* (5bp deletion) and *cx34.1* (8bp deletion; Miller et al., 2017), we examined the localization of Connexin and ZO1 proteins in

mutants (Fig. 6). First, we found that *cx35.5*$^{\Delta 5bp/\Delta 5bp}$ and *cx34.1*$^{\Delta 8bp/\Delta 8bp}$ mutant animals revealed a complete loss of detectable staining for the mutated protein. In addition, there was a failure of the non-mutated Connexin protein to robustly localize to the electrical synapse although low levels of staining were present. This supports an interdependence between the two neuronal Connexins as was shown in Miller et al., 2017. By contrast, ZO1 staining in the single Connexin mutants was robust at the synaptic contact sites with the stereotyped CE appearance, distribution, and position. By comparing the relative ZO1 fluorescence between wildtype and Connexin mutant animals, we found that ZO1 was present at synaptic contacts at approximately half the normal level (Fig. 6B,C,E). When we examined double mutants, we still found that around 10% of ZO1 staining remained (Fig. 6D, E). These results reveal that ZO1b can localize to putative electrical synaptic sites independent of neuronal Connexin proteins. However, loss of the Connexins does impact the amount of ZO1b staining found at CEs suggesting that the Connexins may be stabilizing ZO1b to some extent at the synaptic site once it localizes. Based on these results, I concluded that ZO1 does not require the presence of channel-forming proteins to localize to electrical synapses during synaptogenesis, yet ZO1 is absolutely essential for proper Connexin localization.

ZO1b PDZ1 can directly interact with neuronal Connexins

Since I had observed both colocalization and a hierarchical genetic relationship between ZO1b and the neuronal Connexins, I wanted to investigate the underlying mechanism. Connexin hemichannels are formed by six transmembrane Connexin protein subunits that interact with other proteins via their extra- and intracellular domains (Fig. 7A). ZO1b is an intracellular membrane-associated guanylate kinase (MAGUK) scaffold protein and contains three PSD95/Dlg/ZO1 (PDZ) protein-protein interaction domains that bind to PDZ binding motifs (PBMs) (Zhu et al., 2016). Previous work demonstrated that the C-terminal four amino acids of mouse Cx36 and perch Cx35 compose PBMs that are essential for interacting with the first PDZ domain (PDZ1) of mammalian ZO1 (Flores et al., 2008; Li et al., 2004). Given that the PBM sequence is conserved in zebrafish Cx35.5 and Cx34.1 proteins (Fig. 7B) and the PDZ1 binding pocket is conserved in ZO1b (Fig.

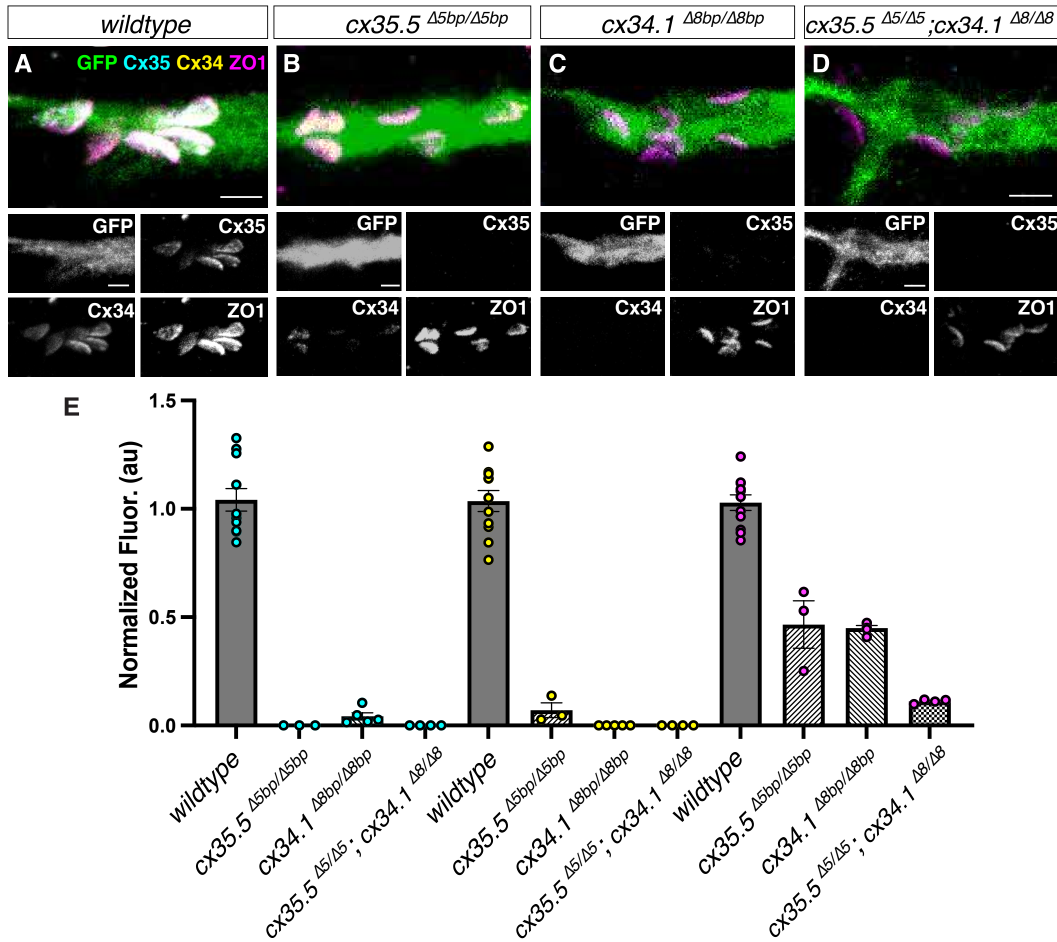


Figure 6. ZO1 localizes at reduced levels to CE synapses in the absence of neuronal Connexins

(A-D) Confocal images of Mauthner lateral dendrite and stereotypical CE electrical synaptic contacts in 5-day-post-fertilization, *zf206Et* zebrafish larvae from *wildtype* (A; combined across three experiments), *cx35.5* $^{\Delta5bp/\Delta5bp}$ (B), *cx34.1* $^{\Delta8bp/\Delta8bp}$ (C), and double mutant (D) animals stained with anti-GFP, anti-zebrafish-Cx35.5, anti-zebrafish-Cx34.1, and anti-mouse-ZO1. Adjacent panels show individual channels. (E) Quantitation of Cx35.5 (cyan), Cx34.1 (yellow), and ZO1 (magenta) at CEs in specified genotypes. Mutants are significantly different than their respective *wildtype* controls in all comparisons (Welch's t-tests, $p<.05$). In Connexin single mutants, where low levels of the other neuronal Connexin remain, about 50% of ZO1 staining remains at CEs. In the Connexin double mutant, only about 10% of ZO1 staining remains at the synapse. The height of the bar represents the mean of the sampled data normalized to the *wildtype* average for a given experiment, and circles represent the normalized value of each individual animal (wt, mut paired experiments: *wt* n=3, *cx35.5*-/- n=3 | *wt* n=4, *cx34.1*-/- n=5 | *wt* n=4, *cx35.5*-/-; *cx34.1*-/- n=4). Error bars are ± SEM.

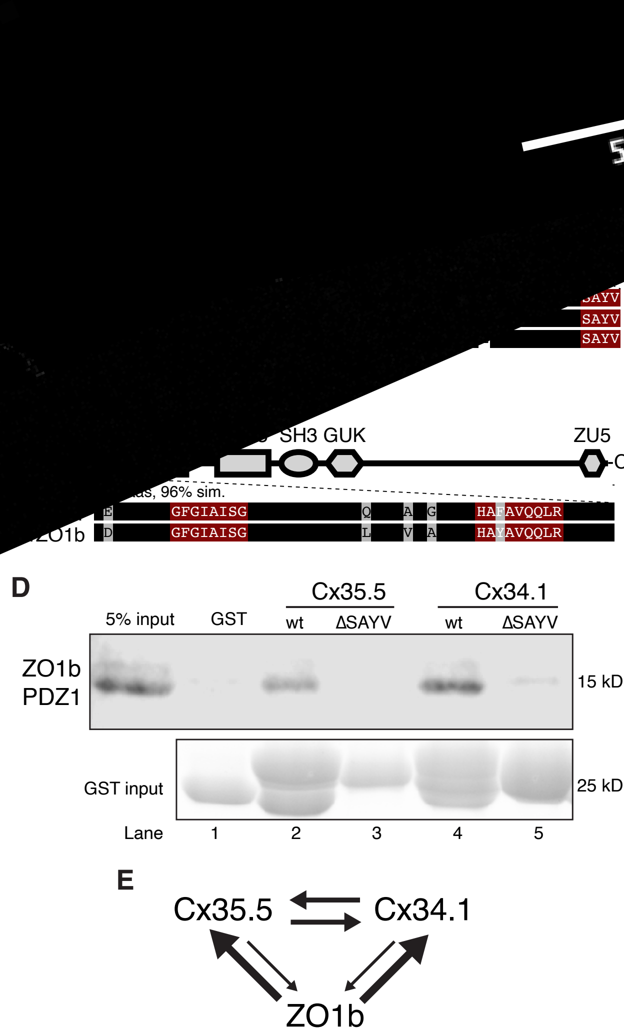


Figure. 7. Direct ZO1b-Connexin interaction may mediate hierarchical organization at CEs
(A) Connexin hemichannels are composed of six Connexin protein subunits that span the membrane. (B-C) Schematic, linear diagrams of Cx36 (B) and ZO1 (C) homologs. Domains are depicted as gray shapes; TM = transmembrane, PDZ, SH3, GUK, and ZU5 = protein-protein interaction modules; hs=Homo sapiens, dr=Danio rerio. Amino acid alignments are shown for the indicated expanded regions. Black bars represent conserved amino acids; non-conserved amino acids are indicated. Maroon boxed amino acids represent the conserved PDZ-binding motif (PBM) of Cx36-family proteins (B) or the predicted PDZ1 residues of the conserved ligand-binding cleft of ZO1-family proteins (C). (D) Bacterially purified GST (lane 1), GST-Cx35.5-tail (lane 2), GST-Cx35.5-tail-ΔPBM (lane 3), GST-Cx34.1-tail (lane 4), or GST-Cx34.1-tail-ΔPBM (lane 5) was immobilized on glutathione beads and incubated with purified ZO1b PDZ1 domain. The tail regions used are depicted in the expanded regions in (B). Bound proteins were analyzed by immunoblot for the presence of ZO1b PDZ1 using anti-TEV cleavage site antibody (top). Equal loading of GST proteins is indicated by protein stain (bottom). (E) Model of molecular hierarchy at the electrical synapse.

7C), we hypothesized that there might be a direct interaction between ZO1b and the neuronal Connexins. I isolated the minimal domains of each zebrafish protein, produced them in bacteria, and performed an *in vitro* binding assay. I found that purified ZO1b PDZ1 could be pulled down with a GST-Cx34.1 or a GST-Cx35.5 C-terminal intracellular-tail (Figure 7D, lanes 2,4), but not with control GST protein (Fig. 7D, lane 1). Additionally, I found that, as expected, the last four amino acids of the Connexin tails were required for interaction to occur (Fig. 6D, lanes 3,5). This shows that the Connexin C-terminal tails are both capable of directly interacting with ZO1b. Therefore, the genetic relationship seen between ZO1b and the neuronal Connexins that it colocalizes with may be due to direct interactions (Fig. 7E).

Discussion

We used forward and reverse genetics to identify ZO1 as a required scaffold at the electrical synapse. In zebrafish, ZO1b, but not ZO1a, was found to be required for CE electrical synapse formation. We showed that this scaffold localized to synaptic sites and was required for robust localization of neuronal Connexins. Furthermore, we showed that ZO1 was able to localize in the absence of Connexin channels suggesting that it may arrive at the synapse first and then promote Connexin localization. Finally, an *in vitro* binding assay revealed that ZO1b can directly interact with both of the neuronal Connexins, thus we hypothesize that ZO1 is promoting Connexin localization through these direct interactions.

These findings force us to update the simplistic model of the electrical synapse in several ways. First, a molecular scaffold is *required* for electrical synapse formation. Work in mammals and fish previously identified ZO1, in addition to several other scaffolds, as localizing to sites of electrical synapse formation (Flores et al., 2008; Li et al., 2004; Li et al., 2012). However, it was never conclusively shown whether these scaffolds were necessary for formation. Our results, in contrast, show that ZO1b is required for Connexin channels to robustly localize. Furthermore, this result replicates what was previously identified by our lab at spinal cord electrical synapses (Marsh et al., 2017) supporting that this is a rule, not an exception. Second, the finding that ZO1b is localizing to electrical synaptic sites in the absence of Connexins suggest that there must

be a molecule upstream, coordinating with the adjacent cell, and telling ZO1b where to make a synapse. We speculate that, as has been shown at chemical synapses, there is a transmembrane cell-adhesion molecule bringing the membranes of the pre- and postsynaptic cells close together, and coordinating recruitment of pre- and postsynaptic machinery, including the scaffold and Connexin channels.

Now that we have shown a scaffold to be required, what might this protein be doing at the synapse? ZO1 is a MAGUK scaffolding protein capable of interacting with cytoskeletal (Fanning et al., 2012) and membrane bound proteins, including Connexin channels (Li et al., 2004; Flores et al., 2008). It has also been found in complexes with CAMKII, a kinase responsible for some forms of electrical synapse plasticity (Alev et al., 2008; Flores et al., 2010; Li et al., 2012). We hypothesize that, given its array of interaction capabilities, it may act as the central organizer at the electrical synapse. Intriguingly, it was recently shown that ZO1 phase separation is necessary for tight junction formation (Beutel et al., 2019; Schwayer et al., 2019). ZO1 oligomerization creates non-membrane bound regions necessary to bring together tight junction machinery. Therefore, it is possible that ZO1 is acting in a similar manner at the electrical synapse.

In the next chapter, I will begin to investigate the mechanisms underlying ZO1s requirement at the electrical synapse. Specifically, I will look *in vivo* to explore the cell biological characteristics of ZO1 and test the requirement of the ZO1-Connexin direct interaction for electrical synapse formation.

Methods

<u>Zebrafish</u>

Fish were maintained in the University of Oregon’s fish facility with approval from the Institutional Animal Care and Use Committee. Zebrafish, *Danio rerio*, were bred and maintained at 28˚C on a 14h on and 10h off light cycle. Animals were housed in groups, generally of 25 animals per tank. Development time points were assigned via standard developmental staging (Kimmel et al., 1995). All fish used for this project were maintained in the ABC background developed at the University of Oregon. Most fish had the enhancer trap transgene zf206Et (referred to as Mauthner:GFP or M/CoLo:GFP) in

the background (Satou et al., 2009), unless otherwise noted. Mutant lines were genotyped for all experiments. All experiments were performed at 5 days post fertilization (dpf). At this stage of development, zebrafish sex is not yet determined (Wilson et al., 2014).

RNA-seq based mutant mapping

As previously described in Miller et al., 2013 and 2017. Briefly, larvae in the F3 generation were collected at 3 dpf from crosses of *dis2* heterozygotes. The posterior portions were fixed for phenotypic identification, and the anterior portions were used for bulk RNA extraction after phenotyping. After creation of cDNA libraries, mapping was performed by identifying high quality 'mapping' single nucleotide polymorphisms (SNPs) in the wildtype pool and assessing these positions in the mutant pool for their frequency. The area of highest average frequency in the mutant pool indicates the region of DNA linked to the phenotype and thus the causative mutation. Within the linked region, candidate mutations causing nonsense or missense changes, or those affecting gene expression levels, were identified as previously described (Miller et al., 2013).

Immunohistochemistry

Anesthetized, 5 dpf larvae were fixed for 3h in 2% trichloroacetic acid in PBS. Fixed tissue was washed in PBS + 0.5% Triton X-100, followed by standard blocking and antibody incubations. Primary antibody mixes included combinations of the following: rabbit anti-human-Cx36 (Invitrogen, 36–4600, 1:200), rabbit anti-Cx35.5 (Fred Hutch Antibody Technology Facility, clone 12H5, 1:800), mouse IgG2A anti-Cx34.1 (Fred Hutch Antibody Technology Facility, clone 5C10A, 1:350), mouse IgG1 anti-ZO1 (Invitrogen, 33-9100, 1:350), mouse anti-RMO44 (Life Technologies, 13–0500, 1:100), and chicken anti-GFP (abcam, ab13970, 1:350- 1:500). All secondary antibodies were raised in goat (Invitrogen, conjugated with Alexa-405, −488, −555, or −633 fluorophores, 1:500). Tissue was then cleared stepwise in a 25%, 50%, 75% glycerol series, dissected, and mounted in ProLong Gold antifade reagent (ThermoFisher, P36930) or 75% glycerol (consistent within experiments).

Imaging

Images were acquired on a Leica SP8 Confocal using a 405- diode laser and a white light laser set to 499, 553/554/557 (consistent within experiments), and 631 nm, depending on the fluorescent dye imaged. Each laser line's data was collected sequentially using custom detection filters based on the dye. Quantitative images of the Club Endings (CEs) were collected using a 63x, 1.40 numerical aperture (NA), oil immersion lens. For each set of images, the optimal optical section thickness was used as calculated by the Leica software based on the pinhole, emission wavelengths, and NA of the lens. Within each experiment where fluorescence intensity was to be quantified, all animals (including 2-5 wildtype controls) were stained together with the same antibody mix, processed at the same time, and all confocal settings (laser power, scan speed, gain, offset, objective, and zoom) were identical. Multiple animals per genotype were analyzed to account for biological variation. To account for technical variation, fluorescence intensity values for each animal were an average across the two Mauthner cells.

Fluorescence quantitation

For the quantitation of staining at the club endings, confocal z-stacks of the Mauthner soma and lateral dendrite were cropped in Fiji (Schindelin et al., 2012) to 36.08μm x 36.08μm centered around the lateral dendritic bifurcation. Using the SciPy (Virtanen et al., 2020) and scikit-image (Van Der Walt et al., 2014) computing packages, the cropped stack was then cleared outside of the Mauthner cell, a 3^3 median filter was applied to reduce noise, and a standard threshold was set within each experiment to remove background staining. The integrated density of each stain within the Mauthner cell was then extracted and normalized to the wildtype values for the experiment.

Means and errors were computed using Prism (GraphPad) or Excel (Microsoft) software. Figure images were created using FiJi and Illustrator (Adobe). Statistical analyses were performed using Prism (GraphPad) and either an unpaired t-test with Welch's correction or a one-way analysis of variance with Bonferrroni's multiple comparison test was performed. For all experiments, values were normalized to *wildtype* control animals, and n represented the number of fish used. Fish were excluded from

analysis if Mauthner morphology/GFP staining was abnormal or if values were greater than two standard deviations away from the mean.

Bacterial expression and purification of proteins

The Cx34.1-tail (aa256-299), Cx34.1-tail ΔPBM (aa256-295), Cx35.5-tail (aa267-309), and Cx35.5-tail ΔPBM (aa267-305) were cloned into the pGEX expression vector allowing for an NH2-terminal GST tag. ZO1b-PDZ1 (aa105-207) was cloned into a modified pET expression vector (pBH) to allow for an NH2-terminal 6xHis tag followed by a TEV cleavage site (vectors kindly provided by Ken Prehoda). Plasmids were transformed in E. coli BL21(DE3) cells and plated on selective LB plates. Single colonies were picked to inoculate 2ml starter cultures and grown overnight. Overnight cultures were inoculated into 250ml selective LB and grown for ~3h at 37°C with shaking until OD600 reached 0.8-1 followed by 4h induction with 0.4mM IPTG. Cell pellets were collected by centrifugation at 6000 RPM for 5min at 4°C and frozen at -20°C until test samples confirmed expression. Pellets were resuspended in sonication buffer (50mM NaPO4 [pH7.4], 300mM NaCl, and 1mM PMSF). After adding a dash of lysozyme, the mixture was incubated on ice for 30min. Resuspended bacteria were sonicated on ice at 50% amplitude, 1sec/1sec pulse on/off, four times for 20sec. Debris was cleared by centrifugation at 16,000 x g for 30min at 4°C. For GST fusions, supernatant was added to 200ul pre-washed glutathione agarose resin and incubated overnight with rocking at 4°C. Beads were washed three times with sonication buffer and stored at 4°C. Purity and amount loaded onto resin was determined by SDS-PAGE followed by Coomassie stain. For 6xHIS fusions, supernatant was brought to a final concentration of 20mM imidazole and incubated with pre-washed His60 resin overnight with rocking at 4°C. Resin was washed with sonication buffer containing 20mM imidazole. Protein was eluted from the resin with sonication buffer containing 250mM imidazole. The protein was concentrated and exchanged into imidazole-free buffer using an Amicon centrifugal filter unit (10K MWCO) and stored at 4°C on ice. Protein concentration was estimated by A205 (https://spin.niddk.nih.gov/clore/)(Anthis and Clore, 2013), and purity was determined by SDS-PAGE followed by Coomassie stain.

In vitro binding assay

Equal amounts of GST fusions (10ul bed of resin) were aliquoted, and the storage buffer was removed. To each sample 15ul of 6xHIS-ZO1b-PDZ1 (7mg/ml) was added, gently mixed and incubated at room temperature for 15min. Resin was washed three times with cold wash buffer (50mM NaPO4 [pH7.4], 300mM NaCl). After the last wash, all buffer was removed, and resin was resuspended in 10ul LDS-PAGE dye with 200mM DTT. Samples were boiled for 3min and resolved by SDS-PAGE using a 4-20% gradient gel. Samples were analyzed by Western blot using rabbit anti-TEV cleavage site primary antibody (ThermoFisher, PA1-119) and visualized with a compatible near-infrared secondary antibody. GST input was visualized by Coomassie to demonstrate equal loading.

CHAPTER IV

ZO1B FACILITATES ELECTRICAL SYNAPSE FORMATION VIA DIRECT INTERACTION WITH THE POSTSYNAPTIC CONNEXIN

All experiments in this chapter were performed, overseen, and/or analyzed by me. I performed the genetics, histology, and imaging with assistance from Jen Michel (Fig. 2). I performed software development and data analysis. I performed the transplant experiments with the help of Adam Miller and Audrey Marsh. Audrey Marsh created the *V5-tjp1b* transgenic zebrafish line. Elisa Trujillo and Jordan Lexa assisted with screening genome-engineered fish. Adam Miller and I conceived of the project and designed experiments. I created the figures and wrote the chapter.

Select data and methods are included in:
Lasseigne AM*, Echeverry F*, Ijaz S*, Michel JC*, Martin EA, Marsh AJ, Trujillo E, Marsden KC, Pereda AE, Miller AC (2021). Electrical synaptic transmission requires a postsynaptic scaffolding protein. *eLife*, 10:e66898 DOI: 10.7554/eLife.668.
* denotes co-first author

Introduction

Electrical synapses are composed of dynamic plaques of Connexin channels that allow direct passage of ions and small metabolites between coupled cells. Each Connexin channel is composed of two hexameric hemichannels contributed by each of the adjoining cells (Goodenough & Paul, 2009). In addition to precise initial localization during synaptogenesis, Connexin proteins turn over roughly every three hours (Flores et al., 2012; Wang et al., 2015) thus requiring complex and ongoing molecular machinery to maintain synaptic integrity and function. One protein known to be involved in this process is the MAGUK scaffolding protein, Zonula occludens-1 (ZO1; Flores et al., 2008; Marsh et al., 2017). Scaffolding proteins function to localize proteins within close proximity with one another to facilitate necessary protein-protein interactions. They often interact with membrane proteins, anchoring them to the cytoskeleton and localizing upstream or downstream molecules. MAGUK proteins, in particular, are a class of

scaffolds whose domain structures allow them to function as master organizers at cellular junctions (Ye et al., 2018). In Chapter III, I showed that ZO1 is necessary for Connexins to localize to sites of electrical synapse formation, but the Connexins are not necessary for ZO1 to localize. This suggests that ZO1 is acting upstream to recruit or stabilize these channel-forming proteins, but the mechanism by which ZO1 facilitates this Connexin localization remains unknown.

At chemical synapses, unique scaffolding proteins are required on either side of these asymmetric structures to organize presynaptic neurotransmitter release and postsynaptic receptor localization (Ackerman et al, 2015; Ye et al., 2018). The asymmetric structure of the chemical synapse combined with the polarized nature of neurons requires that certain scaffolding molecules must be trafficked to and utilized in distinct cell biological compartments. Electrical synapses have classically been assumed to be symmetric, although they too are formed between polarized neurons. Thus, in order for us to understand molecular mechanisms underlying the requirement of ZO1 at the electrical synapse, we must first understand where this scaffold is acting.

To investigate the cell biology of ZO1, we again used the Mauthner cells in larval zebrafish. Zebrafish have two Mauthner cells, one on each side of the body with dendrites and soma located in the hindbrain and an axon descending contralaterally down the length of the spinal cord (Bartelmez, 1933; Kimmel, 1982; Satou et al., 2009). In the hindbrain, auditory afferents make Club Ending (CE) synapses onto the lateral dendrites of Mauthner. In the spinal cord, the Mauthner axon makes segmentally repeating electrical synapses onto Commissural Local Interneurons (CoLos; M/CoLo synapses). Mauthner, therefore, forms electrical synapses in two separate neuronal compartments (i.e., dendrite vs. axon). Although gap junction channels often function homotypically (i.e., the same Connexin hemichannel contributed from each cell), at Mauthner electrical synapses, the Connexins are asymmetric (Miller et al., 2017). Cx35.5 acts presynaptically, while Cx34.1 functions postsynaptically. This provides evidence that electrical synapse formation requires different, compartmentalized mechanisms to make specialized pre- and postsynaptic structures and also serves as an ideal model system to determine whether the asymmetry seen at the level of the Connexins extends to the molecular scaffold.

Here, we utilize the Mauthner morphology to show that ZO1b is localizing and functioning in the postsynaptic compartment. We see evidence of this at both CE and M/CoLo synapses. We then test whether a direct interaction between ZO1b and the postsynaptic Cx34.1 is necessary for electrical synapse formation. We find that their interaction is indeed required *in vivo* for robust Cx34.1 localization to CE, but not M/CoLo, synapses. Electrical synapse components, including the critical scaffold, appear to be compartmentalized, suggesting that cells must have multiple mechanisms to build electrical synapses.

Results

Previous work in zebrafish identified ZO1b, a homolog of mammalian ZO1, to be colocalized with neuronal Connexins at electrical synapses. Cx34.1 and Cx35.5 are channel-forming proteins that require ZO1b for robust synaptic localization and are capable of directly interacting with ZO1b *in vitro* (see Chapter III). In contrast, ZO1b localizes to electrical synapses in the absence of the Connexins, although at lower levels. The genetic and biochemical evidence of the relationship between the Connexins and ZO1b (Fig. 1A, Chapter III) led me to wonder what was happening cell biologically at the synapse. To further investigate the role of the scaffolding protein ZO1b at the electrical synapse, we utilized the electrical synaptic contacts made by Mauthner cells in larval zebrafish.

These synapses contain plaques of Connexin channels composed of asymmetric, interdependent hemichannels. Presynaptic cells use Cx35.5, while postsynaptic cells use Cx34.1 (Fig. 1B; Miller et al., 2017). Zebrafish have two Mauthner cells, one on each side of the body with dendrites and soma located in the hindbrain and an axon descending contralaterally down the length of the spinal cord (Fig. 1C). In the hindbrain, auditory afferents make contacts onto the lateral dendrite of Mauthner, each creating an individual Club Ending (CE) synapse with Mauthner acting as the postsynaptic cell. In the spinal cord, the Mauthner axon makes segmentally repeating contacts with Commissural Local (CoLo) interneurons. At these synapses, which we identify as M/CoLo synapses, Mauthner is acting as the presynaptic cell (Fig. 1D). I examined electrical synapses in

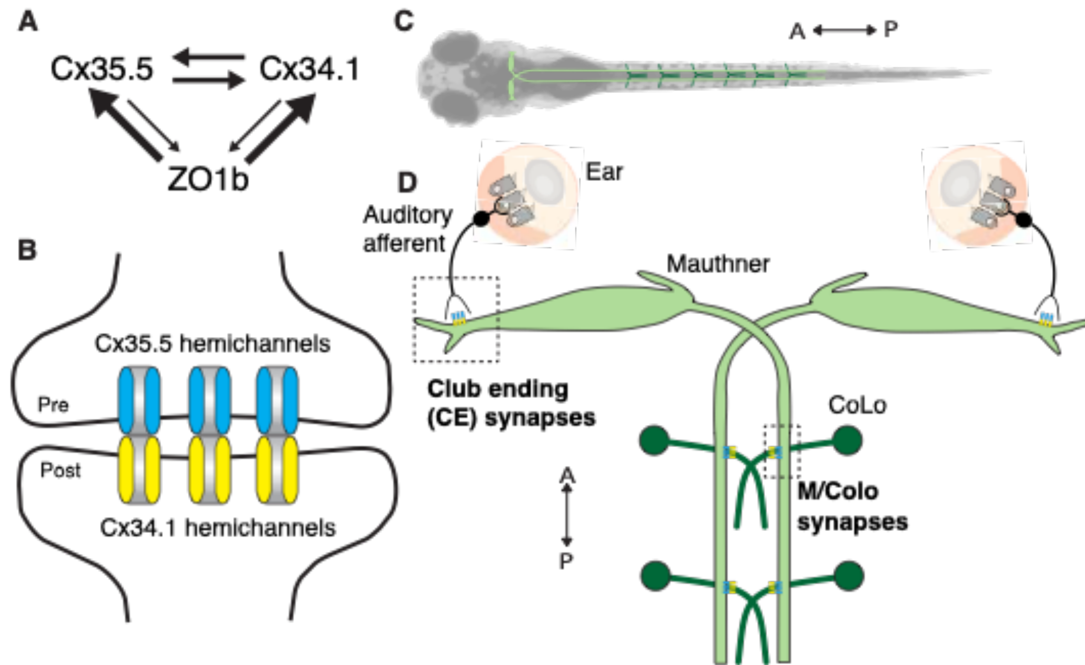


Figure 1. Model of electrical synapses made by Mauthner neurons in zebrafish
Genetic hierarchy (A) and molecular organization of Connexins (B; Cx) at zebrafish electrical synapses. (C) Dorsal view of 5dpf zebrafish larvae (anterior to the left) with Mauthner cells depicted in light green and CoLo interneurons in dark green. (D) Simplified diagram of the Mauthner cell circuit illustrating the electrical synapses of interest. The image represents a dorsal view with anterior on the top. Boxed regions indicate regions of stereotypical synaptic contacts used for analysis. Presynaptic auditory afferents contact the postsynaptic Mauthner cell lateral dendrite in the hindbrain forming Club Ending (CE) synapses. In the spinal cord, the presynaptic Mauthner axons form en passant electrical synapses with the postsynaptic CoLo interneurons (M/CoLo synapses) in each spinal cord hemisegment (2 of 30 repeating spinal segments are depicted). Electrical synapses are denoted as channels (B) or rectangles (D) depicting the two Connexin hemichannels (presynaptic Cx35.5 (cyan) and postsynaptic Cx34.1 (yellow)) that form the neuronal gap junction channels of this circuit.

zebrafish at 5 days post-fertilization (dpf), when there are ~6-9 CEs and ~30 M/CoLos per Mauthner cell.

ZO1b localizes to and acts within the postsynaptic compartment of electrical synapses

Within Mauthner, we know that Cx35.5 is being used in the axon within the presynaptic compartment, while Cx34.1 is being used in the dendrite within the postsynaptic compartment. Therefore, I wondered whether ZO1b, the gene product of *tjp1b*, was also being used in a distinct neuronal compartment. First, I investigated where ZO1b was localizing within the Mauthner circuit. To do this, we developed a V5-tagged

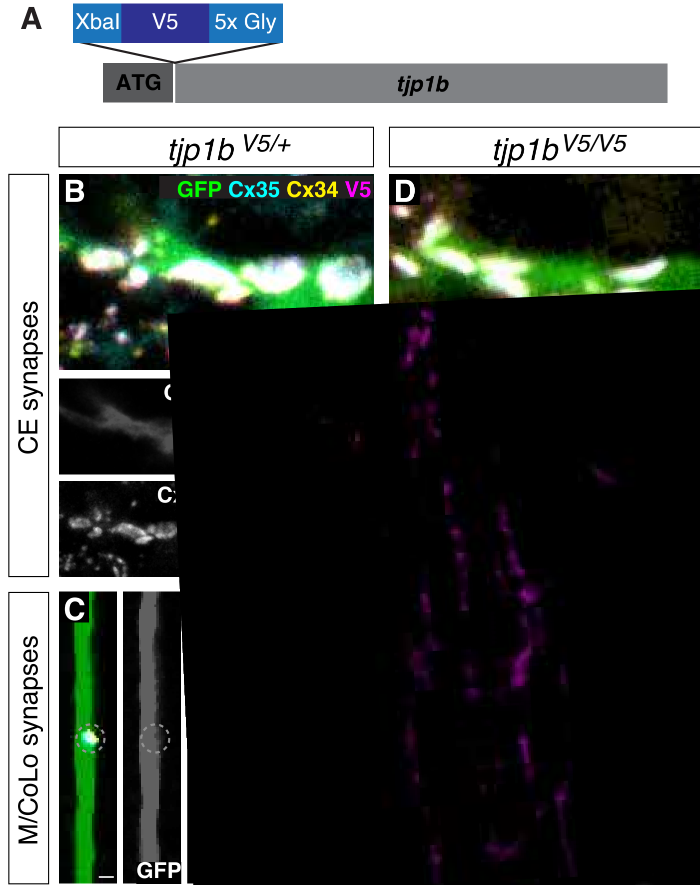


Figure 2. V5-tagged ZO1b localizes to electrical synapses (A) Schematic of V5 epitope tag insertion into the ... of the endogenous ... Confocal image... circuit neurons...

tjp1b allele (Fig...

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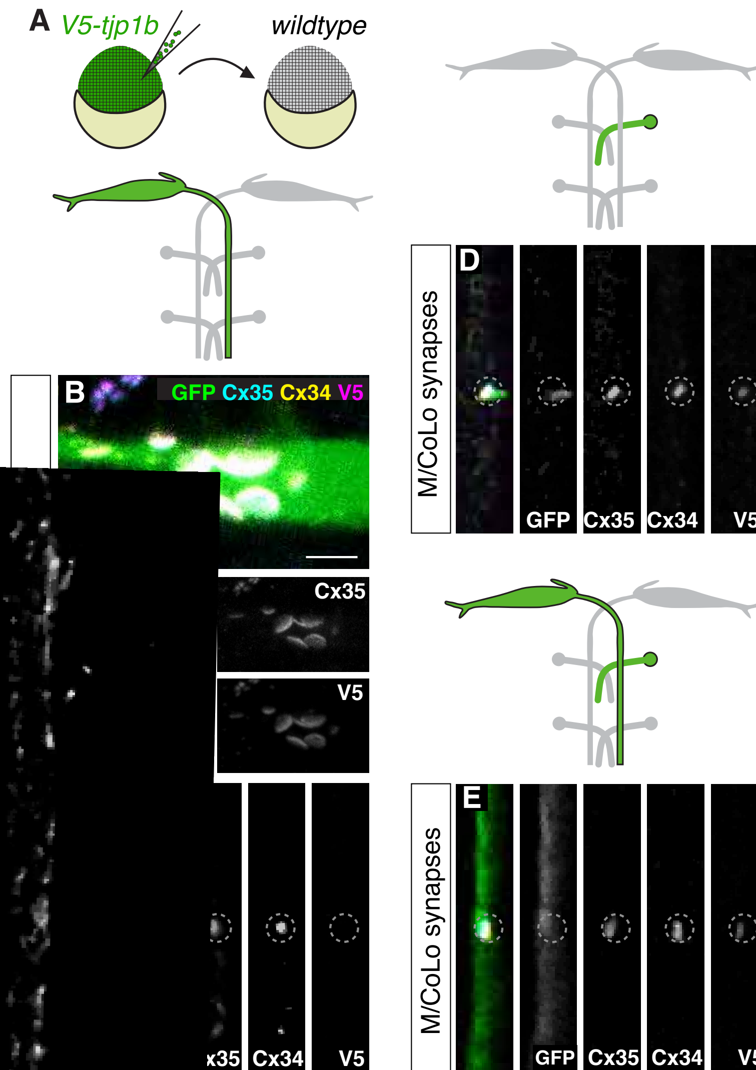


hin the postsynaptic compartment at Mauthner

which GFP-expressing donor cells are transplanted into a
himeric embryos. GFP-expressing cells are either
for V5-tjp1b while the rest of the cells in the chimeric
type. The circuit model depicts the transplanted cells in
al images of CE and M/CoLo sites of contact in 5dpf
of images, GFP-expressing cells (Mauthner (B, C), CoLo
Lo (E)) are derived from a V5-tjp1b+/-; zf206Et donor.
ned with anti-GFP (green), anti-zebrafish-Cx35.5 (cyan),
), and anti-V5 (magenta). Scale bar = 2 µm in all images.
um-intensity projections of ~5 µm. Images of M/CoLo
lices and have been passed through median filter to remove

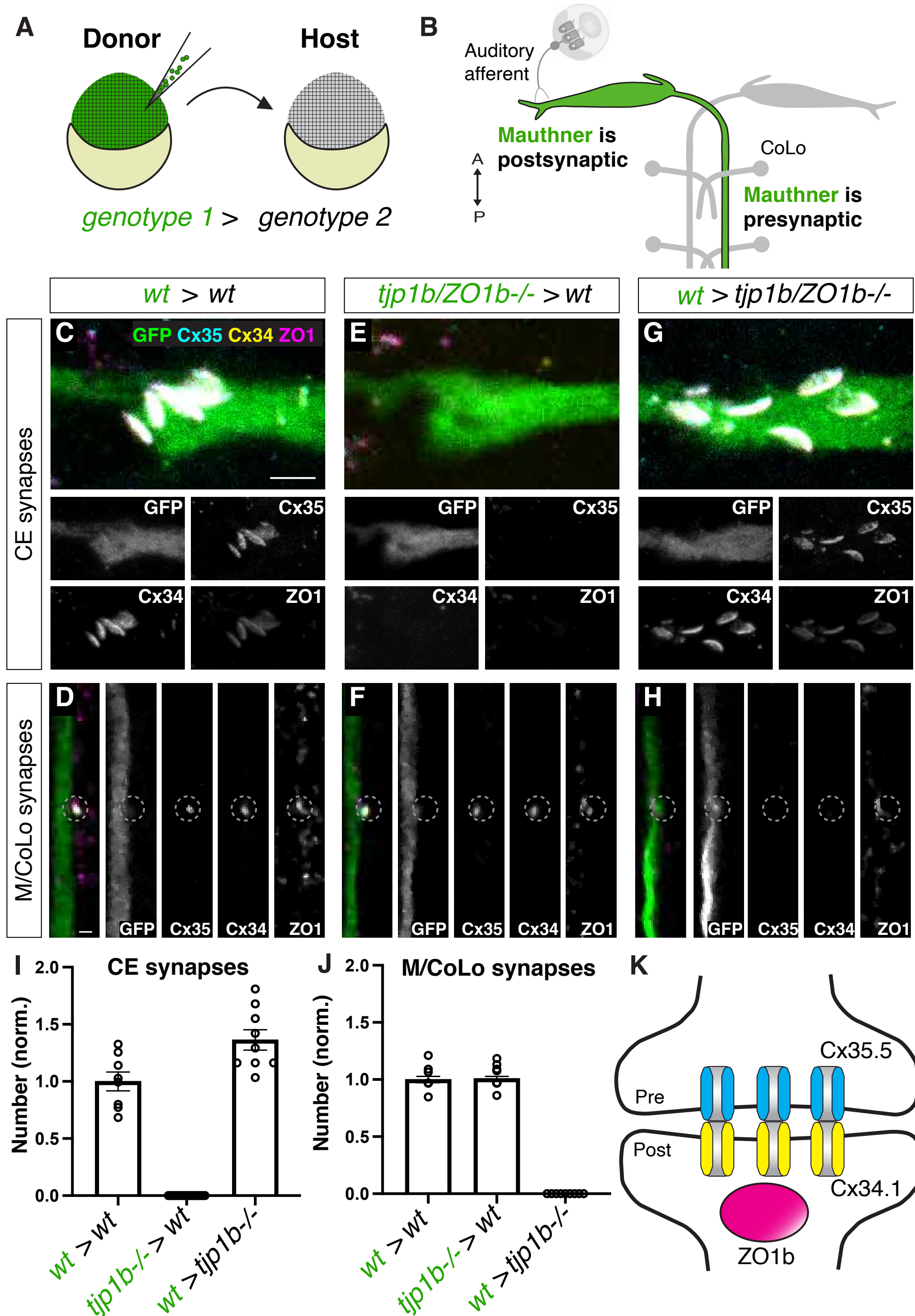
In animals where Mauthner was the only cell expressing *V5-tjp1b*, V5 could be seen at CEs colocalizing with Cx34.1 and Cx35.5. This means that ZO1b is localizing within the Mauthner dendrite and into the postsynaptic compartment of the electrical synapse (Fig. 3B). When I looked at the M/CoLo synapses, however, I saw that V5 was not localizing to electrical synapses, although the neuronal Connexins were present at normal levels. Thus, ZO1b was not localizing within the Mauthner axon nor presynaptically at the electrical synapse (Fig. 3C). Fortuitously, our transplants also created animals in which the only cell expressing *V5-tjp1b* was a CoLo. In such cases, V5 was present at the M/CoLo synapse, again supporting ZO1b's localization within the postsynaptic compartment (Fig. 3D). V5 was also seen at M/CoLo synapses when both Mauthner and CoLo expressed *V5-tjp1b* (Fig. 3E). Taken together, I concluded that ZO1b localizes to the postsynaptic compartment of Mauthner electrical synapses.

I next addressed whether ZO1b functions postsynaptically to facilitate Connexin localization at the electrical synapse. To test for asymmetric requirement, we again utilized chimeric animals and took advantage of Mauthner cell morphology (Fig. 4A, B). However, in these experiments we transplanted GFP-expressing cells from $tjp1b^{\Delta 16bp/\Delta 16bp}$ or *wildtype* donors into $tjp1b^{\Delta 16bp/\Delta 16bp}$ mutant or *wildtype* hosts, producing animals in which ZO1b was specifically removed from the pre- or postsynapse (Fig. 4C-J). At CE synapses, where Mauthner is postsynaptic, removal of *tjp1b* from Mauthner resulted in a loss of Connexin and ZO1 staining (Fig. 4E). In a reciprocal experiment, when Mauthner is the only cell expressing *tjp1b* (i.e., the presynaptic auditory afferents do not produce ZO1b), CE electrical synapses are maintained (Fig. 4G). This suggests that *tjp1b* is required and sufficient in the postsynapse at CEs for cell-autonomous localization of Cx34.1 and cell-non-autonomous localization of Cx35.5. Furthermore, when we looked at M/CoLo synapses in chimeric animals, the same pattern emerged. When ZO1b was removed from the presynaptic Mauthner cell, the M/CoLo staining was maintained (Fig. 4F). However, when only the Mauthner cell contained ZO1b (ie. the postsynaptic CoLos did not), ZO1 and Connexin staining was lost (Fig. 4H). Taken together, these results support that ZO1b is functioning exclusively in the postsynaptic cell where it is localizing (Fig.4K). Interestingly, it appears to be required cell-autonomously for Cx34.1 localization as well as cell-non-autonomously for Cx35.5 localization.

ZO1b-Cx34.1 direct interaction is required for normal electrical synapse formation

My *in vitro* experiments in Chapter III revealed that ZO1b can directly interact with both Cx34.1 and Cx35.5. However, my transplant experiments support a model in which ZO1b is only localizing and acting in the postsynaptic compartment with Cx34.1. Therefore, I hypothesized that the ZO1b-Cx34.1 interaction might be required for electrical synapse formation. ZO1b contains three PSD95/Dlg/ZO1 (PDZ) protein-protein interaction domains that bind to C-terminal PDZ binding motifs (PBMs) (Fig. 5A; Zhu et al., 2016). Each Cx34.1 hemichannel is composed of six Connexin protein subunits, each embedded in the membrane with an intracellular C-terminal tail containing a PBM (Fig. 5B). I have previously shown that the Cx34.1 C-terminal tail can directly interact with PDZ1 of ZO1b in a manner that requires the last 4 amino acids of the tail, SAYV (see Chapter III).

Figure 4 (next page). ZO1b is required and sufficient in the postsynaptic compartment for electrical synapse formation
(A) Diagram of experiment in which GFP-expressing donor cells are transplanted into a non-transgenic host to create chimeric embryos. GFP-expressing cells are of *genotype1* while the rest of the cells in the chimeric embryo are derived from *genotype2*.
(B) Schematic of the Mauthner circuit in chimeric animals where one Mauthner cell is derived from the GFP-expressing donor (green), while other neurons derive from the non-transgenic host (gray). The image represents a dorsal view with anterior to the top. Boxed regions indicate regions imaged for analysis. (C-H) Confocal images of Mauthner circuit neurons and stereotypical electrical synaptic contacts in 5 dpf chimeric zebrafish larvae in the postsynaptic compartment of Mauthner electrical synapses. Animals are stained with anti-GFP, anti-zebrafish-Cx35.5, anti-zebrafish-Cx34.1, and anti-mouse-ZO1. The genotype of the donor cell (green, *genotype1*) and host (*genotype2*) varies and is noted above each set of images (*genotype1 > genotype2*). Images of CEs (C, E, G) are maximum-intensity projections of ~5 µm. Images of M/CoLo synapses (D, F, H) are single Z-slices passed through a median filter. Neighboring panels show individual channels. Scale bar = 2 µm in all images. (I-J) Quantification of the number of stereotypical electrical synaptic structures labeled with both anti-Cx34.1 and anti-Cx35.5 in chimeric animals of the indicated genotypes with a GFP-positive Mauthner cell. The height of the bar represents the mean of the sampled data normalized to the wt>wt average, and circles represent the normalized value of each individual animal (CE synapses: wt>wt n=8, tjp1b/ZO1b-/->wt n=16, wt>tjp1b/ZO1b-/- n=9; M/CoLo synapses: wt>wt n=11, tjp1b/ZO1b-/->wt n=18, wt>tjp1b/ZO1b-/- n=9). Error bars are ± SEM. (K) Schematic summarizing the results of Fig. 3 and Fig.4. ZO1b localizes, is required, and is sufficient exclusively in the postsynaptic compartment of Mauthner electrical synapses.



To investigate whether this interaction is required for electrical synapse formation *in vivo*, I used CRISPR cas9 to disrupt the Cx34.1 PBM sequence. I created two different mutations that would disrupt the ability of the Cx34.1 tail to interact with the ZO1b PDZ1 binding pocket (Fig. 5C). The first mutation, *cx34.1ΔYV,* removes the final two amino

acids of the tail, while the second, *cx34.1+LGA,* adds three new amino acids. Neither mutated tail was predicted to interact with ZO1 PDZ1 or any other PDZ domain (Hui et al., 2010, 2013). Therefore, I have likely disrupted the ability of ZO1b to interact with the Cx34.1 C-terminal tail in these two mutants.

We tested our mutant alleles in animals heterozygous for the *cx34.1Δ8bp* null allele (indicated as -). The C-terminal mutations also disrupted the 3'UTR sequence, so we first ensured that this did not impact electrical synapse formation. *cx34.1 3'UTR* mutants have a 14bp deletion following the stop codon. In animals that are $cx34.1^{+/-}$ or $cx34.1^{3'UTR/-}$, localization of Cx34.1, Cx35.5, and ZO1 to CEs is comparable to *wildtype* (Fig. 5D, G-I). However, in animals where the Cx34.1 protein lacks YV or is extended by LGA, Cx34.1 fails to localize to CE synapses (Fig. 5E, F, H) supporting that the Cx34.1-

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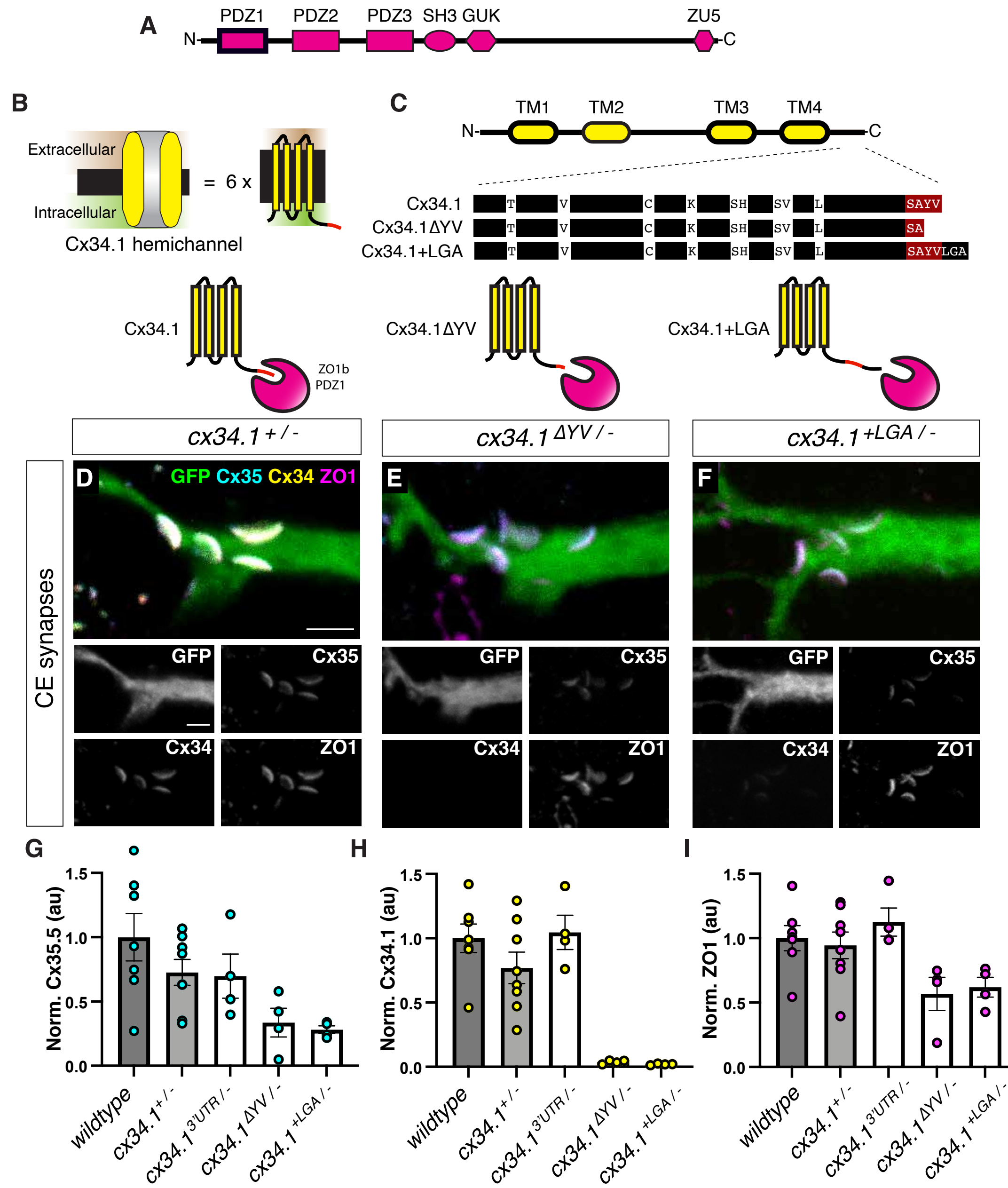
Figure 5 (next page). ZO1b-Cx34.1 interaction is required for Cx34.1 localization at CEs

(A) Schematic, linear diagram of ZO1b (A). Domains are depicted as magenta shapes. PDZ, SH3, GUK, and ZU5 = protein-protein interaction modules. The PDZ1 domain (thick black outline) interacts *in vitro* with Cx34.1. (B) Cx34.1 hemichannels are composed of six Connexin protein subunits that span the membrane and contain a C-terminal PDZ binding motif (indicated in red) known to interact with ZO1 PDZ1. (C) Mutations in the Cx34.1 c-terminal tail were created to test the requirement of the PDZ1-PBM interaction *in vivo*. Schematic, linear diagram of Cx34.1 with domains are depicted as yellow shapes. TM = transmembrane. Amino acid alignments are shown for the indicated expanded regions Cx34.1ΔYV allele removes the last two amino acids of the protein and thus disrupts the PBM. Cx34.1+LGA allele adds three amino acids after the PBM which is hypothesized to disrupt the ability of the PBM to interact with the PDZ binding pocket. (D-F) Confocal images of Mauthner lateral dendrite and stereotypical electrical synaptic contacts in 5-day-post-fertilization, *zf206Et* zebrafish larvae from $cx34.1^{+/-}$ (D), $cx34.1^{\Delta YV/-}$ (E), and $cx34.1^{+LGA/-}$ (F). In all cases, animals are heterozygous for the cx34.1Δ8bp null allele (indicated as -). The second allele is either *wildtype* (D), *cx34.1ΔYV* (E), or *cx34.1+LGA* (F) with the predicted PDZ1 interaction modeled above. When the Cx34.1 PBM is disrupted, Cx34.1 staining at the CEs is lost and Cx35.5 staining is reduced (ANOVA with multiple comparisons, $p<.05$). Animals are stained with anti-GFP (green), anti-zebrafish-Cx35.5 (cyan), anti-zebrafish-Cx34.1 (yellow), and anti-mouse-ZO1. Neighboring panels show individual channels. Scale bar = 2 µm in all images. (G-I) Quantitation of Cx35.5 (G), Cx34.1 (H), and ZO1 (I) at CEs in specified genotypes. The c-terminal mutations also impact the 3'UTR sequence, so we also included $cx34.1^{3'UTR/-}$ in our quantitation as a control. $cx34.1^{3'UTR}$ has a 14bp deletion following the stop codon. The height of the bar represents the mean of the sampled data normalized to the *wildtype* average for a given experiment and circles represent the normalized value of each individual animal (*wt* n=6, *cx34.1+/-* n=7, $cx34.1^{3'UTR/-}$ n=4, $cx34.1^{\Delta YV/-}$ n=4, $cx34.1^{+LGA/-}$, n=4). Error bars are ± SEM.



ZO1b interaction is required for Cx34.1 localization. Additionally, ZO1 levels in *cx34.1^+LGA/-^* animals were decreased by ~50% which is similar to what we see in Cx3 null animals (see Chapter III; ANOVA with multiple comparisons, $p<.05$). This sugg that the dependence of ZO1 on the Connexins is also mediated through this direct interaction. Interestingly, although Cx35.5 localization to the synapse was decreased when ZO1b-Cx34.1 binding was abolished in *cx34.1^+LGA/-^* animals, it was not lost to the

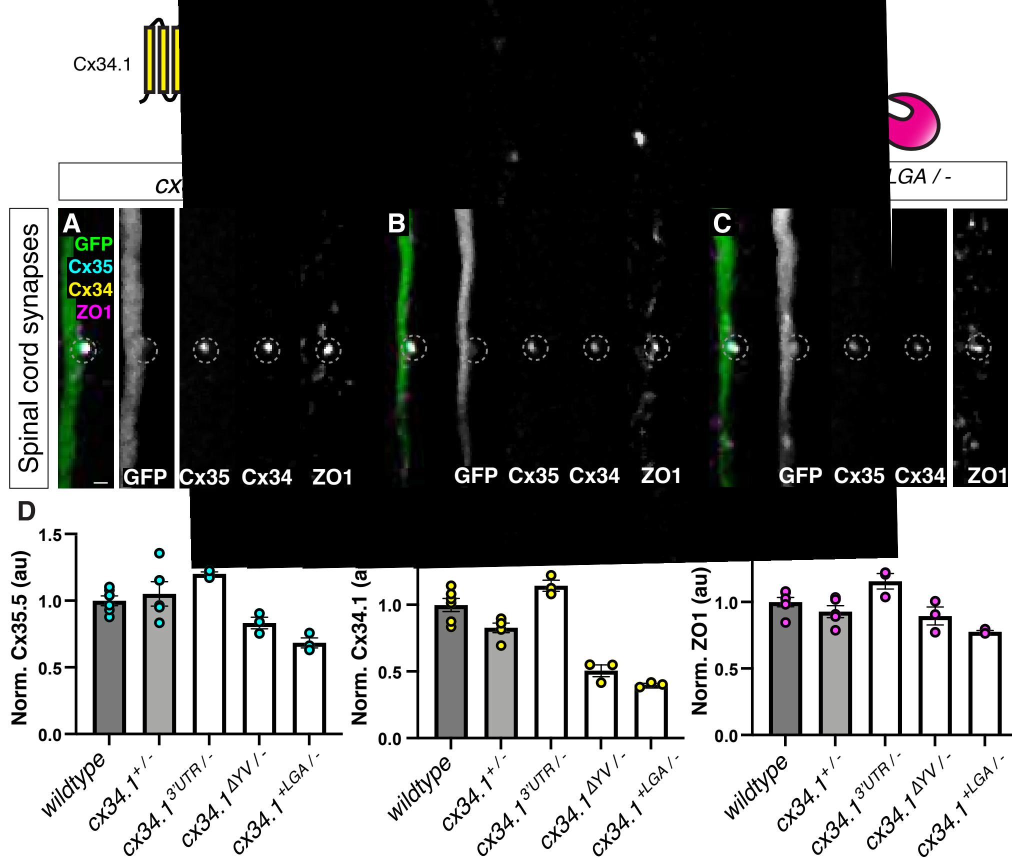


Figure 6. ZO1b-Cx34.1 interaction is not required for robust Cx34.1 localization to M/CoLo synapses
(A-C) Confocal images of Mauthner axon and stereotypical M/CoLo electrical synaptic contacts in 5 dpt, *zf206Et* zebrafish larvae from *cx34.1*$^{+/-}$ (A), *cx34.1*$^{\Delta YV/-}$ (B), and *cx34.1*$^{+LGA/-}$ (C). In all cases, animals are heterozygous for the *cx34.1Δ8bp* null allele (indicated as -). The second allele is either wildtype (A), *cx34.1ΔYV* (B), or *cx34.1+LGA* (D) with the predicted PDZ1 interaction modeled above. When the Cx34.1 PBM is disrupted, Cx34.1, Cx35.5, and ZO1 staining remain at M/CoLos. Images of M/CoLo synapses are single Z-slices passed through a median filter. Animals are stained with anti-GFP (green), anti-zebrafish-Cx35.5 (cyan), anti-zebrafish-Cx34.1 (yellow), and anti-mouse-ZO1. Neighboring panels show individual channels. Scale bar = 2 μm in all images. (D-F) Quantitation of Cx35.5 (D), Cx34.1 (E), and ZO1 (F) at M/CoLos in specified genotypes. The height of the bar represents the mean of the sampled data normalized to the *wildtype* average for a given experiment and circles represent the normalized value of each individual animal (*wt* n=6, *cx34.1+/-* n=5, *cx34.1*$^{3'UTR/-}$ n=3, *cx34.1*$^{\Delta YV/-}$ n=3, *cx34.1*$^{+LGA/-}$, n=3). Error bars are ± SEM.

level that we observe in ZO1b or Cx34.1 null mutant animals (Fig. 5G; see Chapter III; ANOVA with multiple comparisons, p<.05).

We also looked at M/CoLo synapses. Again, in animals that are *cx34.1*$^{+/-}$ or *cx34.1*$^{3'UTR/-}$, localization of Cx34.1, Cx35.5, and ZO1 to M/CoLos is comparable to

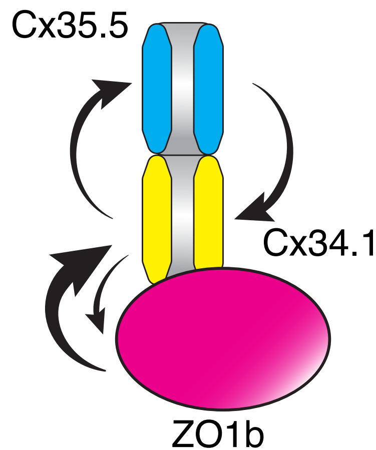


Figure 7. Model of interactions mediating electrical synapse hierarchy at CEs
Presynaptic Cx35.5 and postsynaptic Cx34.1 are genetically interdependent. The scaffold, ZO1b, localizes with Cx34.1 in the postsynapse, is sensitive to the loss of Cx34.1, and is genetically required for both the pre- and postsynaptic Connexins to robustly localize. This is mediated, in part, through a direct ZO1b-Cx34.1 interaction. Arrow thickness indicates strength of genetic relationship.

wildtype (Fig. 6A, D-F). Surprisingly, Cx34.1 was still able to localize to electrical synapses in both *cx34.1*$^{\Delta YV/-}$ and *cx34.1*$^{+LGA/-}$ animals (Fig. 6B-C, E). The loss of the interaction did reduce the amount of Cx34.1 present at the synapse by ~50% (ANOVA with multiple comparisons, $p<.05$) suggesting that, although the ZO1b-Cx34.1 interaction is not required at M/CoLo synapses for Cx34.1 localization, it does impact the amount the Cx. This discrepancy between the CE and M/CoLo synapses was unexpected, and it suggests that there are alternative methods used by cells to build electrical synapses. It is also interesting to note that in ZO1b mutants, Connexin staining at M/CoLo synapses is almost entirely lost. This means that the requirement for ZO1b at these synapses is not mediated entirely through its interaction with Cx34.1.

Discussion

Overall, our findings add to the growing evidence that electrical synapses are far more complex than simple, symmetric plaques of Connexin channels. First, we found that the MAGUK scaffolding protein, ZO1b, localizes to and functions within the postsynaptic compartment of the electrical synapse. Second, we showed that, at CE synapses, ZO1b seems to be directly interacting with the postsynaptic Cx34.1, and this interaction is necessary for the Connexin to localize (modeled in Fig. 7). This supports the idea that electrical synapses can have distinct pre- and postsynaptic specializations,

comparable to the chemical synapse. If this is the case, then cells must have distinct mechanisms in place to build each type of specialization.

Interestingly, when we disrupted the ZO1b-Cx34.1 interaction, the presynaptic Cx35.5 still robustly localized. We previously showed that ZO1b is necessary for the robust localization of the presynaptic Cx35.5, and null mutations in either *cx34.1* or *tjp1b* cause a loss of Cx35.5 at the synapse (see Chapter III). Based on this, we hypothesized that the requirement of ZO1b for Cx35.5 was likely mediated via Cx34.1. However, this does not appear to be the case. One possible explanation is that ZO1b also plays a larger role at the synapse beyond its interaction with the Cx. Indeed, MAGUK proteins often act as master regulators at cellular junctions, and, recently, the ability of MAGUK proteins, to phase separate has been shown to be necessary for tight junction and chemical synapse formation (Ye et al., 2018; Beutel et al., 2019; Schwayer et al., 2019; Chen et al., 2020). However, ZO1b is still postsynaptic, begging the question: how is it coordinating with the presynapse if not through the postsynaptic Connexin?

At the M/CoLo synapse, we were surprised to find that the ZO1b-Cx34.1 interaction was not required for electrical synapse formation. Connexin and ZO1 staining levels appeared lower than in wildtype, but robust staining remained for all proteins. This is one of the first times that we have seen a genetic manipulation that affects the CE and M/CoLo synapses differently. This result suggests that there are multiple, possibly redundant, mechanisms for building an electrical synapse.

Overall, our findings emphasize the molecular complexity of electrical synapses. We previously thought of them at simple aggregates of channels. However, we now know that they asymmetrically require a MAGUK scaffolding protein. We also know that this scaffold is necessary for Connexin localization via a direct interaction with PDZ1. However, this interaction alone does not explain ZO1's entire role at the synapse. We sought to understand the mechanism underlying ZO1's requirement at the synapse, and we successfully uncovered one piece of the puzzle. Future work will assess ZO1's other functions at the synapse, answering questions such as: How is the scaffold localized? What other direct interactions is the scaffold making? Are there other scaffolds acting at and organizing electrical synapses? How is the scaffold coordinating organization of the presynapse?

Methods

Zebrafish

Fish were maintained in the University of Oregon's fish facility with approval from the Institutional Animal Care and Use Committee. Zebrafish, Danio rerio, were bred and maintained at 28˚C on a 14h on and 10h off light cycle. Animals were housed in groups, generally of 25 animals per tank. Development time points were assigned via standard developmental staging (Kimmel et al., 1995). All fish used for this project were maintained in the ABC background developed at the University of Oregon. Most fish had the enhancer trap transgene zf206Et (referred to as Mauthner:GFP or M/CoLo:GFP) in the background (Satou et al., 2009), unless otherwise noted. Mutant lines were genotyped for all experiments. All experiments were performed at 5 days post fertilization (dpf). At this stage of development, zebrafish sex is not yet determined (Wilson et al., 2014). Newly generated lines were created using CRISPR cas9 technology as reported in Shah et al., 2016 with guides designed using the CRISPRscan algorithm (Moreno-Mateos et al., 2015). Mutant animals were Sanger sequenced to verify genomic changes.

Immunohistochemistry

Anesthetized, 5 days post fertilization (dpf) larvae were fixed for 3h in 2% trichloroacetic acid in PBS. Fixed tissue was washed in PBS + 0.5% Triton X-100, followed by standard blocking and antibody incubations. Primary antibody mixes included combinations of the following: rabbit anti-Cx35.5 (Fred Hutch Antibody Technology Facility, clone 12H5, 1:800), mouse IgG1 anti- Cx35.5 (Fred Hutch Antibody Technology Facility, clone 4B12, 1:250), rabbit anti-Cx34.1 (Fred Hutch Antibody Technology Facility, clone 3A4, 1:250), mouse IgG2A anti-Cx34.1 (Fred Hutch Antibody Technology Facility, clone 5C10A, 1:350), mouse IgG1 anti-ZO1 (Invitrogen, 33-9100, 1:350), mouse IgG2a anti-V5 peptide (Invitrogen, R960-25, 1:50), and chicken anti-GFP (abcam, ab13970, 1:350-1:500). All secondary antibodies were raised in goat (Invitrogen, conjugated with Alexa-405, −488, −555, or −633 fluorophores, 1:500). Tissue was then cleared stepwise in a 25%, 50%, 75% glycerol series, dissected, and mounted in ProLong Gold antifade reagent (ThermoFisher, P36930) or 75% glycerol.

Imaging

Images were acquired on a Leica SP8 Confocal using a 405- diode laser and a white light laser set to 499, 553/554/557 (consistent within experiments), and 631 nm, depending on the fluorescent dye imaged. Each laser line's data was collected sequentially using custom detection filters based on the dye. Quantitative images of the Club Endings (CEs) were collected using a 63x, 1.40 numerical aperture (NA), oil immersion lens, and images of M/CoLo synapses were collected using a 40x, 1.20 NA, water immersion lens. For each set of images, the optimal optical section thickness was used as calculated by the Leica software based on the pinhole, emission wavelengths, and NA of the lens. Within each experiment where fluorescence intensity was to be quantified, all animals (including 3-5 wildtype controls) were stained together with the same antibody mix, processed at the same time, and all confocal settings (laser power, scan speed, gain, offset, objective, and zoom) were identical. Multiple animals per genotype were analyzed to account for biological variation. To account for technical variation, fluorescence intensity values of each animal were an average across multiple regions.

Blastula cell transplantation

Cell transplantation was performed at the high stage approximately 3.3 hr into zebrafish development using standard techniques (Kemp et al., 2009). Embryos were chemically dechorionated with protease (Sigma Aldrich, 9036-06-0) prior to transplantation. Cells were transplanted using a 50 mm wide glass capillary needle attached to an oil hydraulic. For '*V5-tjp1b* into *wildtype*' transplants, cells from animals heterozygous or homozygous for *V5-tjp1b* in the M/CoLo:GFP background were transplanted into non-transgenic *wildtype* hosts. For '*tjp1b-/-* into *wildtype*' transplants, genotyped animals homozygous for the *tjp1bΔ16bp* mutation in the M/CoLo:GFP background were crossed and progeny were transplanted into non-transgenic wildtype hosts. For '*wildtype* into *tjp1b-/-*' transplants, transgenic M/CoLo:GFP *wildtype* animals were crossed to use as donors, and non-transgenic, homozygous *tjp1b*$^{\Delta 16bp/\Delta 16bp}$ animals were crossed to produce hosts. Approximately 20 cells were deposited ~10–15 cell diameters away from the margin, with a single donor embryo supplying cells to 3–5 hosts. At 5 dpf, larvae were fixed in TCA and processed for immunohistochemistry.

Fluorescence quantitation

For the quantification of staining at the CEs, confocal z-stacks of the Mauthner soma and lateral dendrite were cropped in Fiji (Schindelin et al., 2012) to 36.08μm x 36.08μm centered around the lateral dendritic bifurcation. Using the SciPy (Virtanen et al., 2020) and scikit-image (Van Der Walt et al., 2014) computing packages, the cropped stack was then cleared outside of the Mauthner cell, a 3^3 median filter was applied to reduce noise, and a standard threshold was set within each experiment to remove background staining. The integrated density of each stain within the Mauthner cell was then extracted and normalized to the wildtype values for the experiment. For quantification of staining at M/CoLo synapses, X and Y coordinates of synaptic sites were manually specified as 9x9 pixel squares. The z-position was determined as the five consecutive slices where the average fluorescence for Cx35.5 was the highest. Within this volume, the average pixel intensity for each channel was quantified and this was averaged across 14-21 synapses per animal. Values per animal were normalized to *wildtype* values for a given experiment.

CHAPTER V
CONCLUSIONS

Electrical synapses are specialized cellular adhesions between neurons that allow ions and small molecules to directly move from one cell to another. Historically, these synapses have been largely neglected in our efforts to understand the brain and are often considered to be simple aggregates of channels. But, in this thesis, I have shown that we need to look beyond the channels. In Chapter II, I considered the biological steps necessary to build an electrical synapse based on what we know about other cellular junctions. Although the channels are the final piece that result in a functional synapse, many steps must occur for those channels to make it to the right place at the right time. Moreover, we know that electrical synapses are dynamic. Connexins are constantly being turned over, and the hemichannels that are at the plaque at any given time are subject to multiple regulatory mechanisms (Nagy et al., 2018; Pereda, 2016; O'Brien, 2017; Lauf et al., 2002; Flores et al., 2012; Wang et al., 2015). Our review of the literature illuminated the many unknowns that still remain in our understanding of the electrical synapse. However, it also led me to hypothesize that a membrane-associated guanylate kinase (MAGUK) scaffold was likely to be necessary at electrical synapses to organize this intercellular structure.

In Chapter III, we used forward and reverse genetics in larval zebrafish to identify the MAGUK scaffolding protein, Zonula occludens-1b (ZO1b), a homolog of mammalian ZO1, as being necessary for electrical synapse formation. This protein localized to electrical synapses and was necessary for robust Connexin channel localization to synaptic sites. In contrast, ZO1b remained at the synapse in the absence of Connexins suggesting a hierarchical organization where ZO1b is localizing first and then recruiting or stabilizing the channel-forming proteins. I also showed that ZO1b can directly interact *in vitro* with the neuronal Connexins through conserved interaction domains. This result, coupled with previous findings of ZO1-Connexin interactions in mammals (Li et al., 2004), led us to hypothesize that ZO1 may be organizing the electrical synapse through this direct interaction with the Connexin channels.

In Chapter IV, we investigated this hypothesis, again, using the larval zebrafish. An examination of the cell biology of the scaffold revealed that it localizes and functions

exclusively in the postsynaptic compartment within our model system. This finding is supported by co-immunoprecipitation experiments showing an asymmetric binding preference between ZO1b and the postsynaptic Connexin (Lasseigne et al., 2021). Therefore, to investigate whether the direct ZO1b-Connexin interaction was the mechanism underlying ZO1's requirement for electrical synapse formation, I used CRISPR cas9 technology to disrupt the region of the Connexin tail responsible for the direct interaction. To our surprise, the mutation of the Connexin tail did not completely abolish electrical synapse formation. In the hindbrain of the zebrafish, we saw a loss of the postsynaptic Connexin suggesting that the interaction is required for its localization. However, the presynaptic Connexin remained, albeit at lower levels. In addition, at synapses in the spinal cord, both Connexins remained at synaptic sites suggesting that, at these synapses, the interaction with ZO1 is not required. These unexpected results suggested that a) ZO1 has multiple functions at the electrical synapse and b) cells use multiple mechanism to form electrical synapses.

I set out to identify mechanisms of electrical synapse formation. Ultimately, I found a molecule that is critical for synapse formation and function which answered some questions but led to many more. Although my work has focused on electrical synapses in zebrafish, it contributes to a growing body of knowledge on a poorly understood synaptic subtype. These structures have diverse functions throughout the mature and developing nervous systems. And, although their impact on disease etiology remains to be thoroughly studied, their unique electrical properties have been shown to influence circuit development, connectivity, and overall function (Nagy et al., 2018; Pereda, 2016; O'Brien, 2017). Therefore, it is imperative that we continue to explore the molecular mechanisms underlying electrical synapses in order for us to truly understand the brain.

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