

SYNCAM2A IS NECESSARY TO DRIVE SYNAPTOGENESIS IN DEVELOPING

DANIO RERIO

By

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Professor Philip Washbourne

Synaptogenesis is a vital process for the development of higher cognitive functions such as learning and memory. When the constructive pathways that lead to synapse formation are interrupted, severe neurological disorders such as autism, schizophrenia, and mental retardation can ensue. It is for this reason that the development of synapses merits further investigation. The aim of this project was to characterize a zebrafish synaptic cell adhesion molecule, SynCAM2a, whose human ortholog has been shown to be important for promoting synapse formation in cultured neurons. To determine the protein's function *in vivo*, SynCAM2a was knocked down with a translation-blocking morpholino and characterized through immunofluorescence and touch response assays. Immunofluorescence showed that SynCAM2a is important for recruiting several pre- and postsynaptic components to the Rohon Beard sensory neuron: Commissural Primary Ascending interneuron synapse, a key constituent of the touch response neuronal circuit. SynCAM2a knock down also showed a 50% reduction in the responsiveness of zebrafish embryos to touch, confirming the necessity of SynCAM2a in constructing this behavioral pathway. Additionally, we found that the intracellular 4.1- and PDZ-binding motifs of SynCAM2a are necessary for promoting synaptic assembly, signifying that both domains are involved in coordinating the recruitment and stabilization of proteins at the pre- and postsynaptic terminals. Together, these findings suggest that SynCAM2a is important for forming the synaptic contacts necessary to develop a functional neuronal network and drive a defined behavior.

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Introduction

Neuroscience

As a whole, the nervous system is quite complex – a single anatomical system which can pattern anything from the most basic of reflex arcs to the sophisticated mental functions that give rise to consciousness. In order to understand the complicated mechanisms by which the nervous system operates, many neuroscientists have adopted a **reductionist** philosophy. By taking this approach, researchers have been able to establish numerous functional divisions within the nervous system, and thus, better direct their mechanistic studies.

In **vertebrates**, the nervous system can be partitioned into the central (CNS) and peripheral (PNS) nervous systems, as depicted in **Figure 1**. The CNS is composed of the brain and spinal cord whereas the PNS is composed of all other nerves and **ganglia** outside of the CNS. Both the CNS and PNS are comprised of billions of **cells** which can be classified as either **neurons** or supporting cells—**glia** (CNS) and **Schwann cells** (PNS). Neurons are responsible for storing and transmitting the electrochemical information of the nervous system, while glia and Schwann cells exist to provide neurons with mechanical and metabolic support¹.

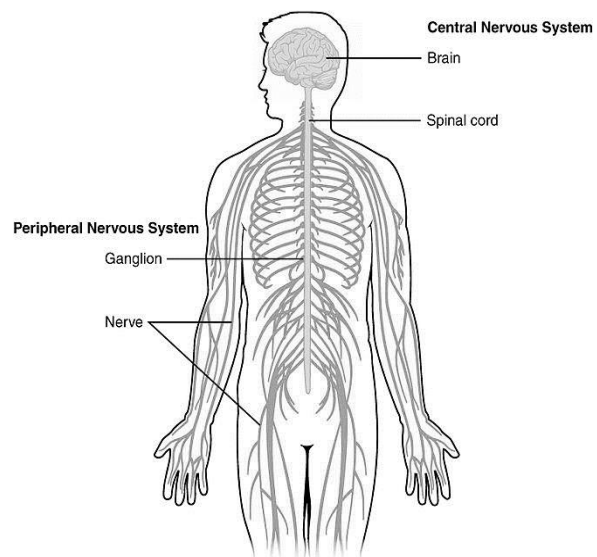


Figure 1. The nervous system². This illustration depicts the central and peripheral nervous systems as well as the units which comprise them (brain, spinal cord, nerves, ganglia).

With over 100 billion nerve cells in the human brain alone¹, it may come as a surprise that each neuron shares a common morphology, as depicted in **Figure 2**. While

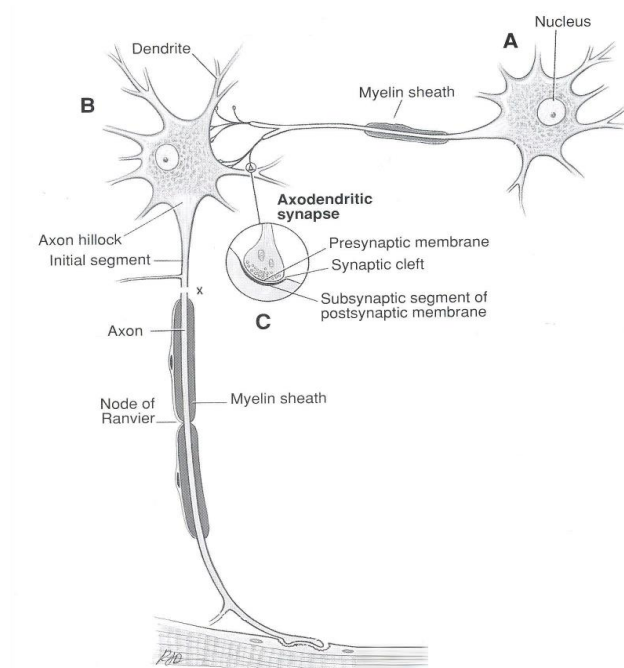


Figure 2. Structure of a neuron³. Labeled features are commonly shared among neurons in the vertebrate nervous system.

the morphology of the supporting cells is more varied, glia and Schwann cells nonetheless perform a conserved set of functions. Specifically, these functions include: surrounding and scaffolding neurons, providing nutrients and oxygen to neurons, insulating dendrites through myelin sheathing, and degrading dead neurons¹.

Although each specific cell type has its own special and unique task, the true computational power of the nervous system is derived from cooperative intercellular interactions, known as **synapses**. At a synapse, two cells, commonly neurons, communicate with each other through either electrical or chemical signals. Although electrical synapses have been shown to play an integral role in numerous neuronal circuits, the vast majority of synapses in adult vertebrates operate via chemical neurotransmission¹.

Chemical neurotransmission is itself dependent upon numerous other biochemical processes, the first of which being **depolarization** of a presynaptic cell. This depolarization occurs as information, in the form of a changing **voltage**, traverses along dendritic arms toward the presynaptic cell body. The axon hillock of the presynaptic cell integrates these voltage changes, and if sufficiently depolarized, dictates the initiation of an **action potential** (AP). Once formed, the AP is propagated along the axon until it reaches the presynaptic terminal. At the presynaptic terminal, the electrical signal of the AP is **transduced** into a chemical signal as voltage-gated calcium ion (Ca^{2+}) channels open. Ca^{2+} influx is detected by presynaptic synaptotagmin **proteins**, which in turn induce a signaling cascade that results in the exocytosis of synaptic vesicles filled with **neurotransmitter** into the synaptic cleft. After diffusing across the synaptic cleft, the neurotransmitters bind neurotransmitter receptors in the membrane of the postsynaptic terminal. This binding event causes either a depolarizing (excitatory) or hyperpolarizing (inhibitory) response in the postsynaptic cell (**Figure 3**). The adult human brain is believed to possess over 100 trillion synaptic connections that function in this manner¹.

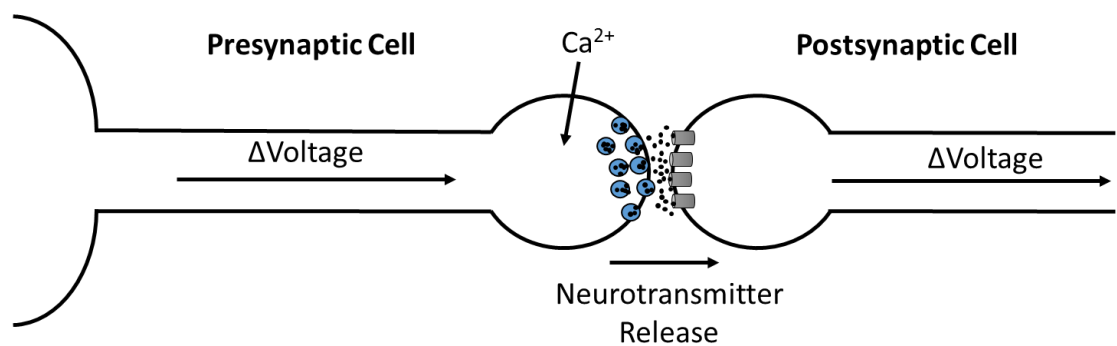


Figure 3. Chemical neurotransmission. Changing voltage in the presynaptic axon leads to calcium influx in the presynaptic terminal. This leads to exocytosis of neurotransmitter into the synaptic cleft, and ultimately, a postsynaptic response (in the form of a voltage change).

Synaptogenesis

The formation of synapses, or **synaptogenesis**, is the focal study of the Washbourne Lab here at the University of Oregon. As previously indicated, a synapse can be defined as the junction between a presynaptic neuronal axon and its postsynaptic partner—typically a dendrite or cell body⁴. All neuronal networks depend upon the proper formation and maturation of synapses at appropriate time points. Moreover, each synapse itself requires a variety of components working harmoniously to form a complex, functional structure⁵. The absence of, or deficiency in, any number of these key components can lead to compromised cognitive function. As such, several developmental and cognitive disorders have been linked to alterations in synaptic composition⁶. Utilizing several molecular biology techniques, our lab hopes to elucidate the events which immediately precede initial contact of synaptic partners through the development and maturation of a synapse.

In order to forge specific synaptic connections, numerous molecular processes must first occur. Synapses are thought to form when the axonal growth cone of a presynaptic cell is directed to the dendrite of its postsynaptic partner cell through chemotaxic attraction⁷. Cell adhesion molecules (CAMs) then help to establish initial contact between synaptic partners, and in turn, initiate the signaling cascade for synaptic formation. This process requires both changes in cell shape and structure at the terminals, as well as recruitment of specialized proteins to form the synapse⁸. In the presynaptic terminal, synaptic vesicles filled with neurotransmitter must be recruited and docked to the plasma membrane, or **active zone**, for subsequent exocytosis in response to stimuli. At the postsynaptic terminal, a multitude of receptors and signaling molecules—

colloquially known as the postsynaptic density—must be assembled in order to convert the neurotransmitter signal into a postsynaptic response⁴. Depending on the identity and concentration of the neurotransmitters present at the synapse, the postsynaptic cell will generate responses of varying intensity as recognized by receptors and propagated by

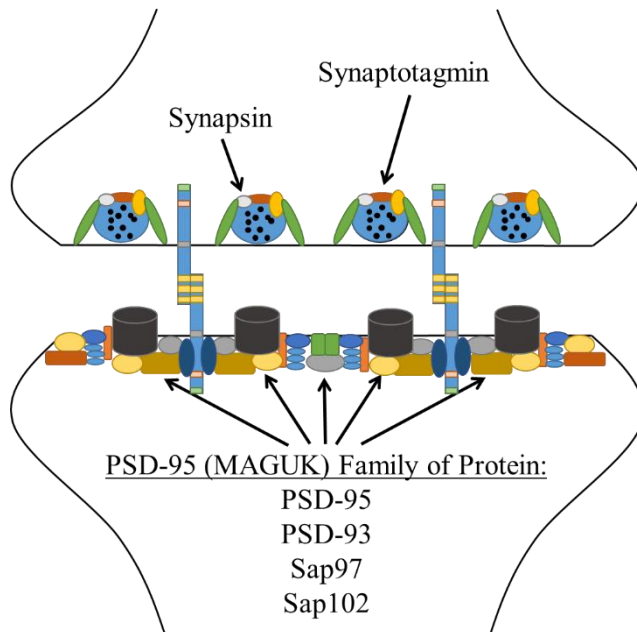


Figure 4. Structure of a synapse. Schematic representation of proteins found at the active zone and in the postsynaptic density. The labeled proteins were used as markers for immunofluorescent experiments to identify synapses.

other postsynaptic, scaffolding, matrix-forming proteins like PSD, Shank, and Homer⁹. In addition to the proteins specific for each terminal, numerous other scaffolding molecules, ion channels, and assorted components must be recruited to the synapse through modular transport packets^{10,11}, as shown in **Figure 4**.

All of these processes rely extensively upon intercellular interactions.

Synaptic Cell Adhesion Molecules

The formation of a mature synapse depends heavily on the establishment of a tight and precise attachment between pre- and postsynaptic cells⁸. Various CAMs which span the synaptic cleft have been implicated in establishing this alignment and attachment. These proteins include neurexins, neuroligins, **immunoglobulin** (Ig) like-domain containing proteins, receptor phosphotyrosine kinases, receptor phosphotyrosine phosphatases, and

several leucine-rich repeat proteins^{10,12}. Of these proteins, a particular group of Ig like-domain containing proteins, called the SynCAM protein family, have been found to be expressed in developing neurons and promote synapse formation^{4,13}.

SynCAM family proteins are known to possess a total of three extracellular, Ig-like domains—which are responsible for cell adhesion—and two intracellular domains known as the 4.1- and PDZ-binding motifs—which facilitate synaptic assembly¹⁴. In **tetrapod** vertebrates, four such SynCAMs (1-4) have been identified and characterized. These SynCAMs are capable of forming both **homophilic** and **heterophilic** complexes across the synaptic cleft. In mice, SynCAM1, 2, and 3 have been shown to form homophilic interactions, while SynCAM1 and 2, as well as SynCAM3 and 4, have also been shown to assemble into heterophilic complexes¹³. In addition to trans-synaptic

organization, SynCAM family members are capable of clustering inside of a single terminal to form cis-conformations¹⁵, as depicted in **Figure 5**. Together, these features of SynCAM family members have significant implications in terms of their ability to complement and compensate for one another.

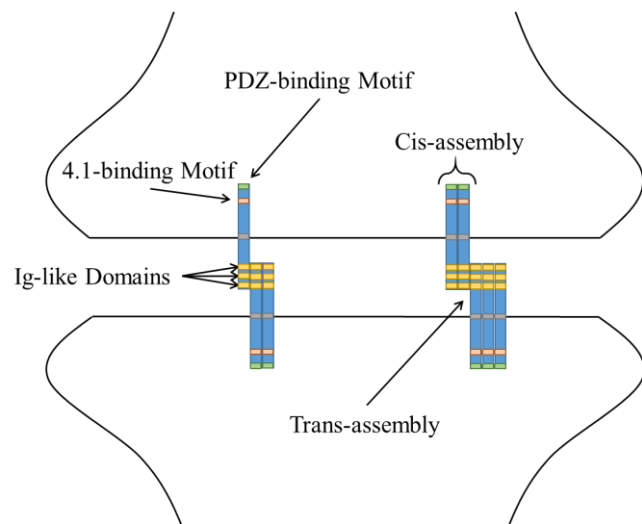


Figure 5. SynCAM structure and assembly. Highlighted domains include the Immunoglobulin (Ig)-like domains, 4.1-binding motif, and PDZ-binding motif. Cis-assembly within a terminal and trans-assembly across terminals are also depicted.

Mutations in SynCAM proteins have been linked to numerous neurodevelopmental disorders, such as autism spectrum disorder (ASD), mental retardation, and schizophrenia^{12,16,17}. With ASD in particular, it is believed that slight alterations in neuronal circuitry and signaling can lead to the impaired social interactions, communication deficiencies, and repetitive behaviors commonly associated with the disease^{16,17}. Incidentally, mutations in the Ig3 domain of human *SynCAM1* have been implicated in the pathogenesis of ASD¹⁷. These **mutant** proteins exhibited defective trafficking to the cell membrane and increased rates of degradation and cleavage¹⁷. Additionally, neurons harboring mutations in *SynCAM1* displayed truncated dendrites¹⁸. Ultimately, these findings indicate that mutations in SynCAM family proteins can lead to the alterations in neuronal circuitry commonly observed in neurodevelopmental dysfunctions. Additionally, they sparked a need for *in vivo* modeling of SynCAM family mutants.

Zebrafish as a Model Organism

In the 1970s, zebrafish (*Danio rerio*) became established as a powerful genetic tool for studying vertebrate development thanks in part to the UO's very own George Streisinger. Although the zebrafish was used as a developmental and **embryological** model as early as the 1930s¹⁹, it was Streisinger's development of numerous genetic techniques that ultimately popularized the **model organism**. By the 1990s, techniques in cloning, **mutagenesis**, **transgenesis**, mapping approaches, and large-scale genetic screens for mutants had underpinned the use of zebrafish as a mainstream model in developmental biology¹⁹.

Desire for establishing the zebrafish model arose from its inherent biologic and genetic benefits. To begin, zebrafish are vertebrates, making them more suitable for human-related studies than other commonly used invertebrate systems, such as *Drosophila melanogaster* and *Caenorhabditis elegans*. Zebrafish can also be bred with relative ease when compared to most vertebrate models. Whereas biologists must wait for many vertebrates to develop inside of the mother's uterus, zebrafish embryos can be immediately and directly accessed; females lay large quantities of eggs in a single setting which are externally fertilized by the males. The absence of the placenta not only eases the observation of early developmental phenomena, but it also greatly increases the number and types of genetic manipulations possible during embryonic stages. In addition to *ex utero* development, zebrafish remain optically transparent for the first 72 hours post-

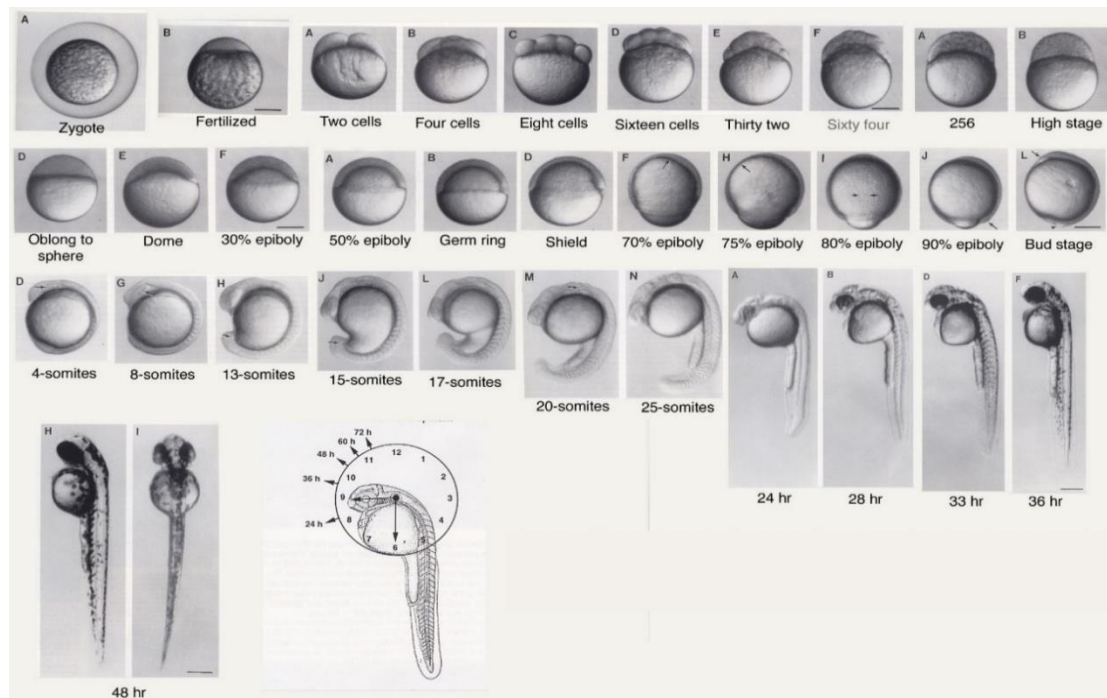


Figure 6. Developmental stages of embryonic zebrafish²⁰. Note the translucent nature of the zebrafish embryo. This unique characteristic permits researchers to easily monitor early developmental processes.

fertilization (hpf) (**Figure 6**), thus allowing researchers to study developmental events from the one-cell stage. Finally, zebrafish develop much faster than most other vertebrate models. They begin to resemble adult morphology by 24 hpf and reach sexual maturity by 10-12 weeks of age¹⁹.

Although only four *SynCAM* **genes** exist in mice and humans, evolutionary processes have bestowed zebrafish with six *SynCAM* **orthologs** (*1a, 1b, 2a, 2b, 3, 4*). This increase in gene number has been attributed to a genome-wide duplication event which occurred after teleost fish branched off from other vertebrates some 110 million years ago²¹. The coding sequences of the zebrafish orthologs are highly conserved with the *SynCAM* family members of tetrapod species and display the same protein domain organization, suggesting that their functions are also conserved²². All six of the zebrafish *SynCAM* genes exhibit dynamic, but highly regulated, expression patterns during development of the CNS. In particular, *in situ* hybridization studies have shown that one of the zebrafish orthologs, *SynCAM2a*, is exclusively expressed in Rohon-Beard (RB) sensory neurons – a primary cell type involved in the embryonic touch response²².

The Touch Response Neuronal Circuit

In addition to the more universal benefits held by zebrafish, their simple and well-characterized nervous system has proven advantageous for our neurologic studies. Prior to 27 hpf, zebrafish exhibit only two marked behaviors: spontaneous tail coiling beginning at 17 hpf and touch-evoked responses beginning at 21 hpf²³. Spontaneous coiling is the first coordinated motor behavior in zebrafish and is the foundation from

which several other behaviors are derived²⁴. The movement is driven by electrical couplings within the spinal cord and is independent of supraspinal input²³. As the embryo matures, these couplings become uncoupled and spontaneous coiling gives way to other unique behaviors such as the touch and startle responses^{23,24}.

As depicted in **Figure 7**, RB sensory cells initiate this circuit by synapsing *en passant* on the cell bodies of Commissural Primary Ascending (CoPA) interneurons. In the touch response, RB cells are responsible for transducing tactile information into an electrical signal that can be used by the neuronal circuit. While spontaneous coils are also believed to utilize this circuitry, they do not require the same tactile stimulation to initiate the circuit. CoPA cells continue the pathway by projecting their axons in the **contralateral** dorsal longitudinal fascicles toward the rostral portion of the spinal cord.

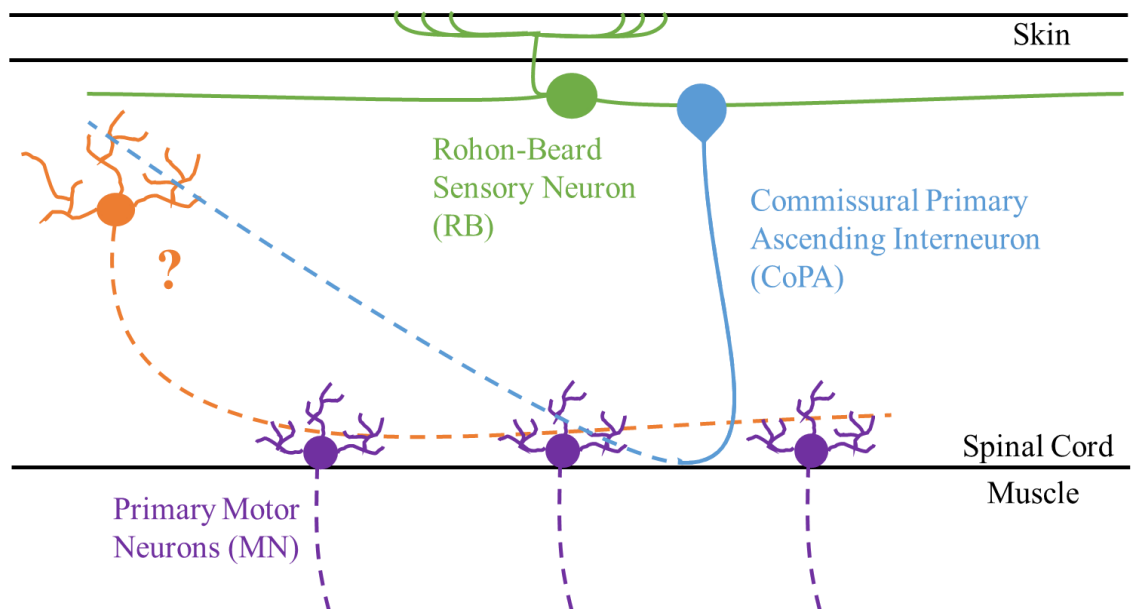


Figure 7. Schematic representation of the touch response neuronal circuit. Solid axonal lines indicate ipsilateral projections while dashed lines indicated contralateral projections. For simplicity, only one RB:CoPA junction is shown.

From there, the exact pathway is still disputed, but it is believed that the CoPA axons connect through both **gap junctions** and **glutamatergic** synapses to **ipsilateral** projecting interneurons and **circumferential** descending interneurons. These descending interneurons are then believed to form connections, via gap junctions and chemical synapses, with ipsilateral motor neurons to invoke muscle contractions²³.

Although previously described as a single, contained circuit, the touch response has evolved mechanisms to amplify its signal. Single RB cells can synapse onto multiple, or even all, CoPA cells on one side of the spinal cord²³. Moreover, CoPA cells are believed to receive input from many, if not all, RB cells on one side of the spinal cord. Ultimately, this divergence and convergence of inputs suggests that stimulation of any single RB will lead to multiple firing of CoPA cells, and thus, efficacy of the response²³.

Project Overview

One of the chief problems that clinicians face in treating neurodevelopmental diseases is the exceptionally complex and poorly-understood nature of the nervous system. Many questions still surround diseases such as ASD and mental retardation because a complete understanding of the brain, from cellular to systemic level, has not been established. This project aims to contribute to the plight of neuroscientists and medical practitioners the world over in comprehending neuronal function by analyzing a fundamental building block required for synaptic transmission.

In zebrafish, a SynCAM family protein, SynCAM2a, was found to be expressed in the well-characterized touch response neuronal circuit. If SynCAM family proteins are indeed important for proper synaptic function, the absence of SynCAM2a should lead to a marked **phenotype** in zebrafish. Moreover, this phenotype should be visible both molecularly, at the RB:CoPA synapse, and behaviorally, in the touch response behavior.

Materials and Methods

Overview

One of the most common and well-established ways that researchers study gene function is **knocking out** or **knocking down** a gene of interest. By examining how an organism functions without expression of a particular gene, researchers can begin to postulate its function. If the gene is then reintroduced into the system, and the **wild-type** phenotype is restored, it can be concluded that loss of the gene, and not some off-target effect, likely caused the observed phenotype.

In this project, SynCAM2a knockdown was achieved through embryonic injection of a translation-blocking **morpholino**. This method of knockdown acts by inhibiting a key step involved in the central dogma of Biology. The dogma states that every organism's genetic code is stored in the form of nucleic acids (typically DNA), and that this code serves as the template from which every organism's physical characteristics are derived. Before these physical characteristics can be developed however, two steps must first occur: the DNA code must be transcribed into a messenger molecule in the form of mRNA, and the mRNA must then be translated into functional protein. By introducing an oligonucleotide (morpholino) into the cell which binds complimentary to *SynCAM2a* mRNA, we were able to effectively block the latter of these two steps. Consequently, we were able to prevent the production of functional SynCAM2a protein in injected embryos.

Once knockdown was established, **immunofluorescent staining** (IF) and touch response experiments were performed to quantify the molecular and behavioral effects

respectively. IF is a common analytical technique used to determine the expression patterns of proteins. It works by exploiting the principle that naturally produced **antibodies** can specifically recognize and bind **antigens** (typically proteins) in biological tissues. Various techniques can then be used to visualize the presence or absence of said antibodies, and consequently, the proteins of interest.

After establishing molecular phenotypes with IF, a touch response **assay** was performed to characterize behavioral phenotypes. All procedures were carried out in compliance with the guidelines of the University of Oregon and IACUC.

Morpholino Knockdown

Knockdown of SynCAM2a was established via a translation blocking morpholino (2a MO) designed anti-sense to *SynCAM2a* mRNA. All injections were performed at the one-cell stage and at a volume of 2 nL. Injections were carried out in a transgenic zebrafish line, *et101.2:Gal4:UAS:GFP*, which drives green fluorescence in RB cells. A control morpholino (control MO) – designed by Gene Tools – and three rescue constructs – full-length *SynCAM2a* RNA (FL), *SynCAM2a* RNA with a deletion in the PDZ-binding motif (Δ PDZ), and *SynCAM2a* RNA with a deletion in the 4.1-binding motif (Δ 4.1) – were also made. All rescue RNAs were cloned into the pXT7 vector.

Injection solutions were made as follows. SynCAM2a knockdown contained 0.8 mM 2a MO and 1/10 volume phenol red. Full-length rescue injections contained 0.8 mM 2a MO, 40 ng/ μ L FL, and 1/10 volume phenol red. Δ 4.1 rescue injections contained 0.8

mM 2a MO, 40 ng/ μ L Δ 4.1, and 1/10 nM phenol red. Δ PDZ rescue injections contained 0.8 mM 2a MO, 40 ng/ μ L Δ PDZ, and 1/10 volume phenol red. Control injections contained 0.8 mM control MO and 1/10 volume phenol red.

After injections, embryos were placed at 28°C and were subjected to a 14 hour on/10 hour off light cycle for a duration of 24 hours. Once 24 hpf, the embryos were dechorionated using surgical forceps and separated for IF and the touch response assay.

Immunofluorescent Staining

Con-1 & Znp-1

Embryos were placed in 1.5 mL Eppendorf tubes and anesthetized with 1 ml of a 0.1% tricaine solution. After removing excess liquid, the embryos were **fixed** with 1 mL of a 4% paraformaldehyde and 1X fish fix buffer (4% sucrose, 0.15 mM CaCl₂, 0.1 M PO₄ buffer at pH 7.3)²⁵ solution overnight on a gentle shaker at 4°C. The embryos were then washed with two changes of PBST (PBS, 0.1% Triton X100) for five minutes each and blocked with PBST/2% normal goat serum (NGS) for one hour on a shaker at room temperature. After blocking, embryos were incubated at 4°C overnight in 200 μ L of primary antibody solution (1:500 chicken anti-GFP, 1:150 mouse IgG1 anti-con-1, and 1:750 mouse IgG2a anti-synaptotagmin2b dilutions in blocking solution). Primary antibody solution was removed and the embryos were washed three times for five minutes, followed by three times for 20 minutes, with PBST. The embryos were subsequently incubated in the dark at 4°C overnight in 200 μ L of secondary antibody

solution (1:500 anti-Ck 488, 1:500 Ms IgG1 633 and 1:500 Ms IgG2a 546 dilutions in blocking solution). Secondary antibody solution was removed and the embryos were washed three times for five minutes, followed by three times for 20 minutes, with PBST. Following staining, embryos were stored in 80% glycerol at 4°C in a foil covering to minimize **photobleaching** from exposure to light.

Synapsin1,2 & PanMAGUK

Embryos were placed in 1.5 mL Eppendorf tubes and anesthetized with 1 ml of a 0.1% tricaine solution. After removing excess liquid, the embryos were fixed with 1 mL of a 4% paraformaldehyde and 1X fish fix buffer solution for 90 minutes on a gentle shaker at 4°C. The embryos were then washed with two changes of PBST for ten minutes and blocked with PBST/2% NGS for one hour on a shaker at room temperature. After blocking, embryos were incubated at 4°C overnight in 200 µL of primary antibody solution (1:500 chicken anti-GFP, 1:1000 rabbit anti-synapsin, and 1:100 mouse anti-panMAGUK dilutions in blocking solution). Primary antibody solution was removed and the embryos were washed three times for five minutes, followed by three times for 20 minutes, with PBST. The embryos were subsequently incubated in the dark at 4°C overnight in 200 µL of secondary antibody solution (1:500 anti-Ck 488, 1:500 anti-rb 633, and 1:500 anti-Ms Cy5 dilutions in blocking solution). Secondary antibody solution was removed and the embryos were washed three times for five minutes, followed by three times for 20 minutes, with PBST. Following staining, embryos were stored in 80% glycerol at 4°C in foil covering.

Imaging

Confocal microscopy was used to obtain all IF images. This imaging technique uses point illumination and a spatial pinhole to eliminate out-of-focus light on tissue samples. In doing so, serial optical selections from thick specimens can be obtained with high resolution and contrast. This is particularly beneficial for fluorescent specimens in which conventional wide-field optical microscopes yield interference from out of focus secondary fluorescence. Additionally, confocal microscopy can be used to reconstruct three-dimensional structure from successive scans of two-dimensional planes²⁶.

Our images were taken on an inverted Nikon TU-2000 microscope with a 60x water immersion objective. The Nikon EZ-C1 confocal programming was used to enhance image precision. Images were taken with a medium pinhole size and laser intensities of 35%, 45%, and 30% for 488 nm, 546 nm, and 633 nm lasers respectively. Image channels were arranged such that the 488 nm, 546 nm, and 633 nm lasers corresponded with green, magenta, and cyan staining respectively. Gain for each channel was modified based upon observable staining but was kept constant throughout individual experiments. Gain levels were optimized such that structural information, but not saturation or excessive noise, was observed.

Images were taken from somites 14-16 of the notochord in sagittally-oriented whole-mount embryos. Upon detection of a CoPA cell body, a **Z-stack** of 6-10 μm with 0.4 μm steps was established. This ensured that information from all points of contact between RB axons and CoPAs was obtained. Images were acquired for each channel separately. Images for Z-stacks were averaged three times with a pixel dwell of 2.16 μm

to improve resolution and reduce background noise. Images were saved as .idf files, a file-type specific for the Nikon EZ-C1 confocal programming. This protocol was repeated for both synapsin1,2/panMAGUK and con-1/znp-1 stained embryos.

Quantification

The following protocol was adapted from Ippolito et al.²⁷. After obtaining .ids files for each experimental condition, the EZ-C1 Free Viewer programming was used to volume render each image. Volume rendering entails converting the Z-stack to a single flattened, maximum intensity projection. These images were saved as RGB .tiff files and opened with ImageJ version 1.26. Using the circular selection tool, a region of interest (ROI) of approximately one cell diameter radially around the soma of interest was selected. With the ROI selected, the ‘Puncta Analyzer’ plugin was launched. This plugin was written by Bark Wark and is available upon request (ceroglu@cellbio.duke.edu). In the ‘Analysis Options’ window that appears, the ‘Red Channel’ (con-1/znp-1 and synapsin1,2/panMAGUK images), ‘Blue Channel’ (synapsin1,2/panMAGUK images only), first ‘Subtract Background,’ and third ‘Subtract Background’ were selected. In the window that appears next, a rolling ball radius of 50 was selected and the white background option was unselected. After selecting ‘OK,’ the threshold slider in the new window was adjusted until the red mask corresponded as well as possible to as many discrete individual **puncta** without introducing excessive noise. This was one of the most subjective steps in the protocol, so special care was taken to develop a consistent approach. In the next window, a minimum puncta size of 4 pixels was selected and all

other options were left with the default values. The previous steps were then repeated with the blue channel for synapsin1,2/panMAGUK images. After completing these steps, the plugin provided quantification corresponding to puncta in each channel as well as colocalized puncta for synapsin1,2/panMAGUK images. This information was exported to Microsoft Excel and two-tailed unpaired *t*-tests were used to test the significance between data sets.

Touch Response Assay

The following assay was adapted from experiments performed by Pietri et al.²³. Mechanical stimulation of the touch response was performed using an insect pin attached to a micromanipulator. Light touches were applied to the sagittal axis of the embryo between somites 14-16, near the caudal portion of the yolk extension. Each trial consisted of four to six stimulations on free-moving embryos, with a spacing of at least one second between stimulations. The absence of each tail contraction to tactile stimulation was recorded and reported as a percentage of total number of stimuli for each fish (failure rate). Two-tailed unpaired *t*-tests were used to test the significance between data sets.

Results

SynCAM2a Clusters Synaptotagmin 2B at Presynaptic Terminals

Following experimental injections, several embryos were stained for synaptotagmin 2B (as detected by Ms IgG1 anti-znp-1) and clathrin light chains a/b (as detected by Ms IgG2a anti-con-1). In the presynaptic terminal, synaptotagmin proteins function as regulators of synaptic vesicle release. These proteins are involved in the docking of synaptic vesicles to the presynaptic membrane^{28,29}, as well as the subsequent exocytosis of said vesicles in response to calcium influx^{30,31}. As such, staining for synaptotagmin 2B serves as an indicator for mature synapses capable of neurotransmission. Clathrin is a protein which functions in the formation of coated vesicles³². Three heavy chains provide structural support for the protein lattice while three separate light chains facilitate the formation and disassembly of the clathrin protein. Specific **epitopes** of clathrin light chains a/b are recognized by the con-1 antibody and can be used to label CoPA cells³³. Localization of znp-1 puncta within con-1 staining therefore indicates the junction between a presynaptic RB axon and a postsynaptic CoPA cell body.

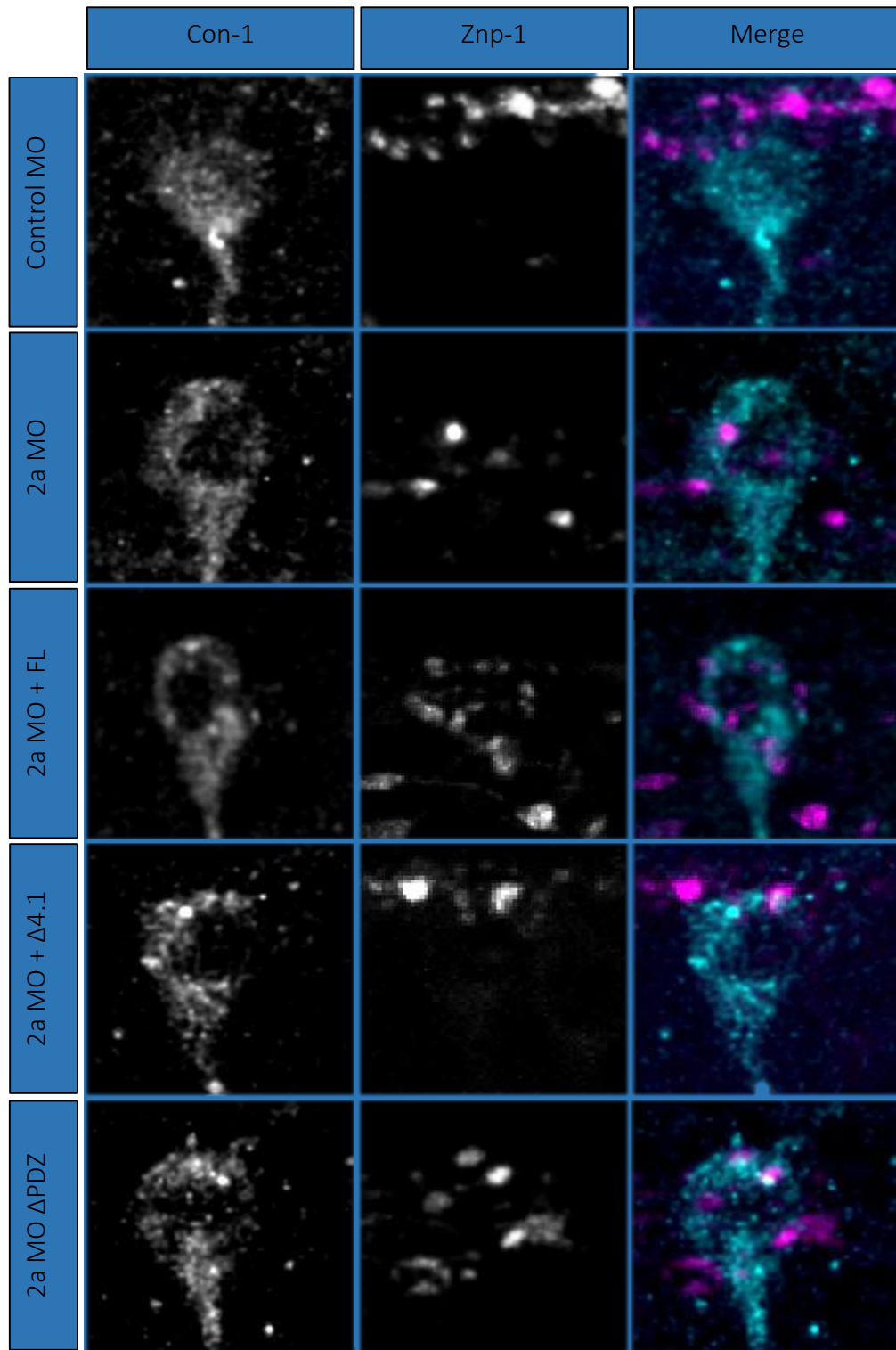


Figure 8. Characteristic con-1/znp-1 staining. Individual channels are presented with white puncta to enhance contrast. In the merged channel, cyan staining indicates the location of con-1 antibody (CoPA cell) and magenta staining indicates the location of znp-1 antibody (synaptotagmin 2B protein). On average, embryos injected with 2a MO, 2a MO + $\Delta 4.1$, and 2a MO + ΔPDZ showed significantly less znp-1 staining within synaptic proximity of CoPA cell bodies than fish injected with Control MO or 2a MO + FL.

Figure 8 depicts characteristic znp-1/con-1 staining for each of the experimental conditions. On average, staining for synaptotagmin 2B within synaptic proximity of CoPA cell bodies decreased by 38 percent after injection with 2a MO (**Figure 9**). Embryos coinjected with 2a MO and full-length *SynCAM2a* RNA exhibited synaptotagmin 2B expression resembling that of control MO injected fish. Neither of the RNA deletion constructs, $\Delta 4.1$ and ΔPDZ , were able to raise synaptotagmin staining levels to that of control MO-injected fish when coinjected with 2a MO. Moreover, coinjection of these rescue constructs with 2a MO did not significantly increase synaptotagmin expression (43 and 30 percent reductions in staining respectively) above embryos injected with 2a MO alone. These findings suggest that *SynCAM2a* is necessary to recruit synpatotagamin 2b to the presynaptic terminal and that both the 4.1- and PDZ-binding motifs are essential for this function of *SynCAM2a*.

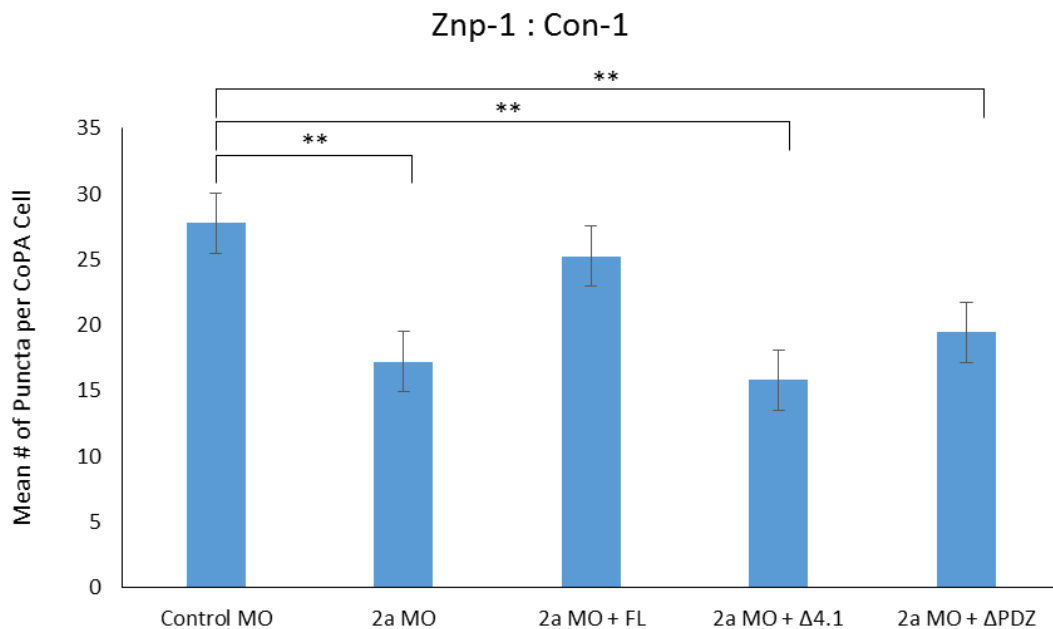


Figure 9. Analysis of *con-1/znp-1* immunofluorescence. Bars indicate the number of *znp-1* puncta localized within synaptic proximity of CoPA cell bodies (as determined by *con-1* staining). Control MO, N = 13; 2a MO, N = 13; 2a MO + FL, N = 12; 2a MO + $\Delta 4.1$, N = 11; 2a MO + ΔPDZ , N = 13. ** indicates a p-value of <0.01. Bars indicate standard error.

SynCAM2a Clusters Synapsin and MAGUK Proteins at Excitatory Synapses

When quantifying synapse number through post-mortem staining, as was done in this study, it is ideal to have both pre- and postsynaptic markers. Although synaptic proteins eventually localize to the terminals in which they function, it is possible to find them outside of the synapse. At any given time, synaptic proteins can be found in the soma (during synthesis) or throughout the neuron's axons and dendrites (while being trafficked to their final destinations). Colocalization of pre- and postsynaptic markers therefore gives a better representation of synapse number because of the decreased likelihood in observing colocalized punctae in locations other than the synapse. Due to the variability in antibody efficacy with respect to tissue fixation however, this cannot always be achieved, as was the case with con-1/znp-1 staining. Fortunately, this study was also able to employ parallel staining for synapsin1,2 (a presynaptic marker) and panMAGUK (a postsynaptic marker).

After completing con-1/znp-1 staining, several embryos were stained for synapsin proteins 1 and 2 (as detected by Rb anti-synapsin) and the PSD-95 family of proteins (as detected by Ms anti-panMAGUK) in order to quantify synapse number. Synapsins are neuron-specific phosphoproteins which have been implicated in the regulation of neurotransmitter release at the pre-synapse. Specifically, these proteins have been shown to play an important role in regulating the releasable pool of synaptic vesicles, clustering synaptic vesicles at active zones, and recycling synaptic vesicles during neurotransmitter release^{34,35}. Consequently, synapsin1 and 2 serve as specific markers for presynaptic terminals throughout the nervous system.

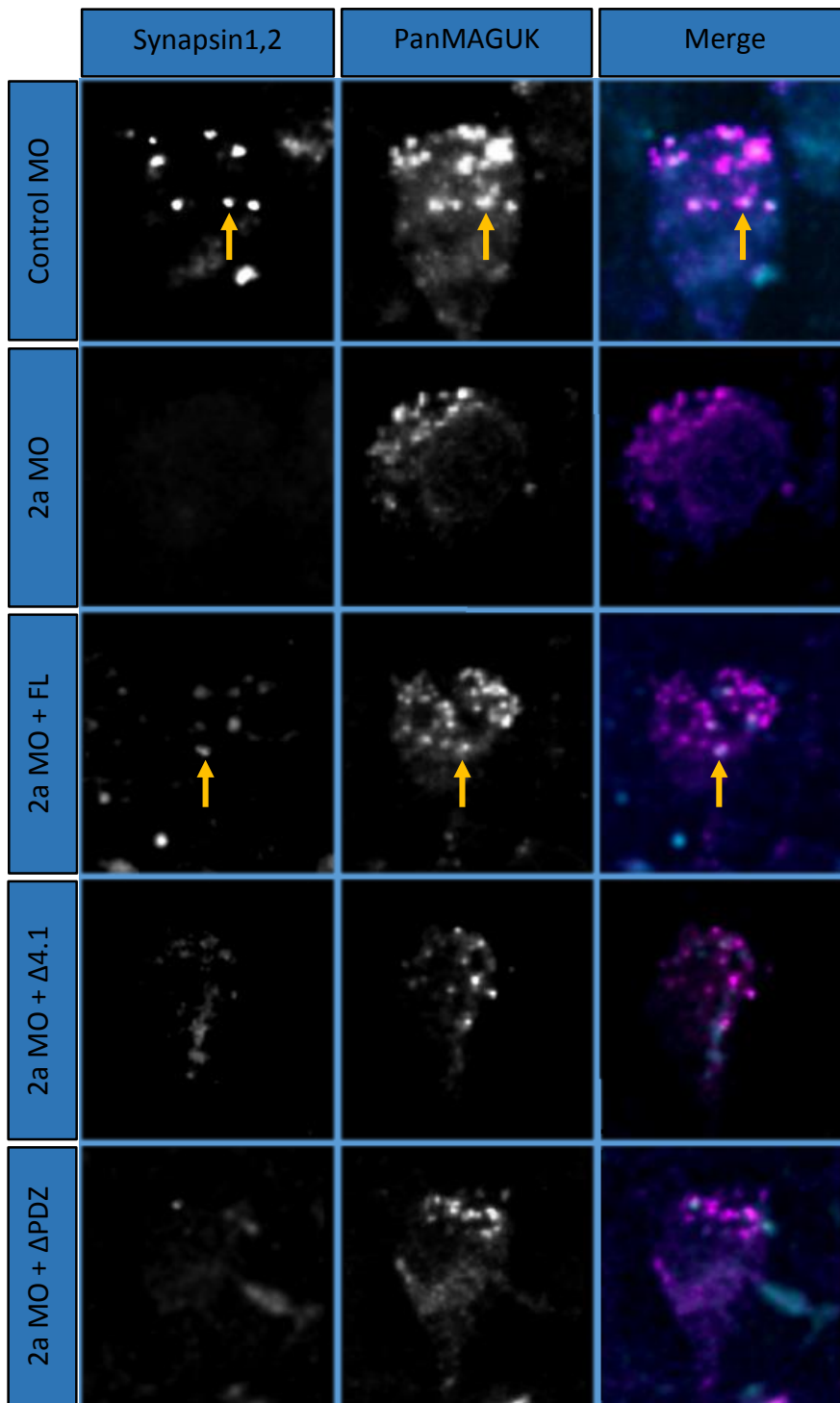


Figure 10. Characteristic synapsin1,2/panMAGUK staining. Individual channels are presented with white puncta to enhance contrast. In the merge channel, cyan staining indicates the location of synapsin1,2 antibody (synapsin proteins 1 and 2) and magenta staining indicates the location of panMAGUK antibody (MAGUK family proteins). Sites of colocalized puncta, as indicated by the arrows, illustrate the location of a synapse. On average, embryos injected with 2a MO, 2a MO + $\Delta 4.1$, and 2a MO + ΔPDZ showed significantly less synapsin1,2, panMAGUK, and colocalized staining than fish injected with Control MO or 2a MO + FL.

The MAGUK (membrane-associated guanylate kinase) proteins are a large family of scaffolding proteins which are characterized by PDZ, SH3, and GUK domains. MAGUK proteins have been implicated in stabilizing synaptic junctions by interacting with cytoskeleton proteins as well as molecules involved in signal transduction³⁶. The anti-panMAGUK antibody recognizes several MAGUK proteins specific to postsynaptic membranes³⁷. Simultaneous labeling of synapsin1,2 and panMAGUK therefore allows detection of not only pre- and postsynaptic structures, but also predicts the location of mature synaptic junctions with high confidence.

Figure 10 displays characteristic synapsin1,2/panMAGUK staining for each of the experimental conditions. Areas of colocalization between the puncta of each channel indicate the location of a synapse. Embryos injected with 2a MO displayed a 48 percent reduction in synapsin1,2 staining within RB axons, a 27 percent reduction in panMAGUK staining within CoPA cell bodies, and a 64 percent reduction in colocalized puncta (**Figures 11-13**). Embryos coinjected with 2a MO and full-length *SynCAM2a* RNA displayed no significant difference in staining for synapsin1,2, panMAGUK, or colocalized puncta compared to control MO injected embryos. Embryos coinjected with 2a MO and Δ 4.1 RNA exhibited a 65 percent reduction in synapsin1,2 staining, a 25 percent reduction in panMAGUK staining, and a 70 percent reduction in colocalized puncta. Embryos coinjected with 2a MO and Δ PDZ RNA exhibited a 54 percent reduction in synapsin1,2 staining and a 74 percent reduction in colocalized puncta, but no significant reduction in panMAGUK staining. Together, these findings suggest *SynCAM2a* is important for coordinating the recruitment of synapsin proteins to the presynaptic terminal, MAGKUK proteins to the postsynaptic terminal, and alignment of

each terminal to one another. Moreover, it can be concluded that both the 4.1- and PDZ-binding motifs are required to facilitate the recruitment of synapsin proteins to the presynaptic terminal, that the 4.1-binding motif is required to facilitate the recruitment of MAGUK proteins to the postsynaptic terminal, and that both motifs are required to coordinate the alignment of the terminals.

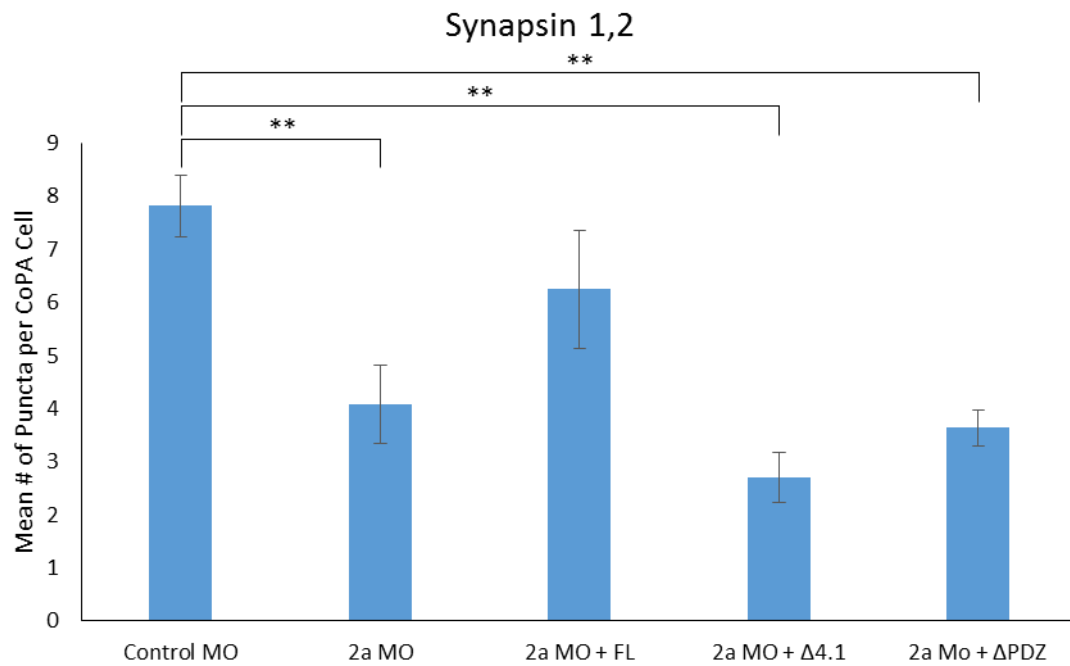


Figure 11. Analysis of synapsin1,2 immunofluorescence. Bars indicate the number of synapsin1,2 puncta localized within synaptic proximity of CoPA cell bodies (as determined by et101.2 GFP). Control MO, N = 11; 2a MO, N = 12; 2a MO + FL, N = 4; 2a MO + Δ4.1, N = 10; 2a MO + ΔPDZ, N = 11. ** indicates a p-value of <0.01. Bars indicate standard error.

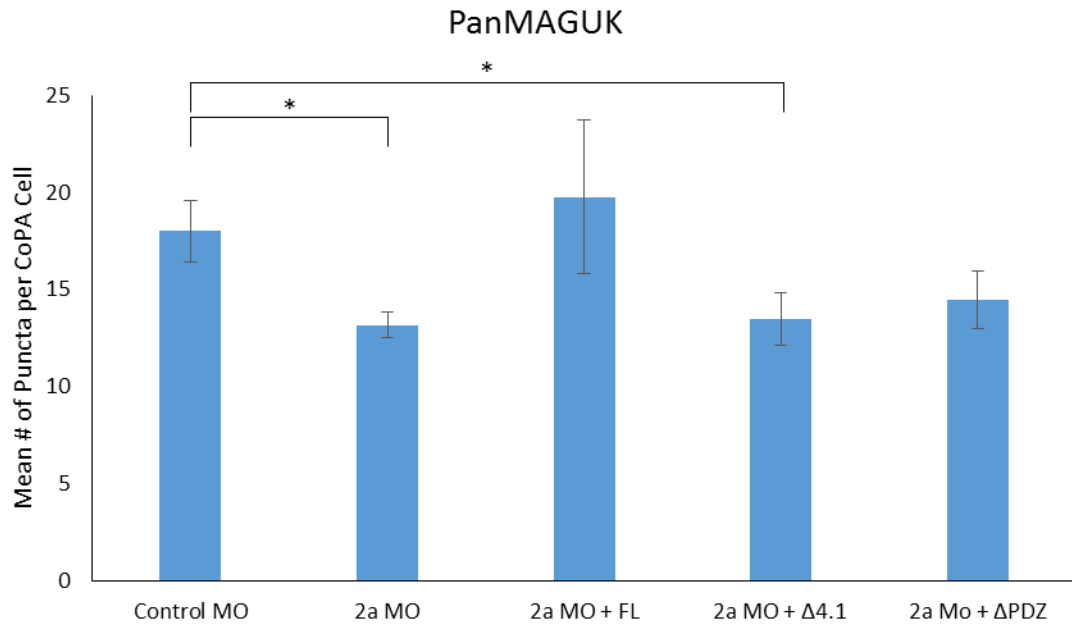


Figure 12. Analysis of panMAGUK immunofluorescence. Bars indicate the number of panMAGUK puncta localized within CoPA cell bodies (as determined by et101.2 GFP). Control MO, N = 11; 2a MO, N = 12; 2a MO + FL, N = 4; 2a MO + Δ4.1, N = 10; 2a MO + ΔPDZ, N = 11. * indicates a p-value of <0.05. Bars indicate standard error.

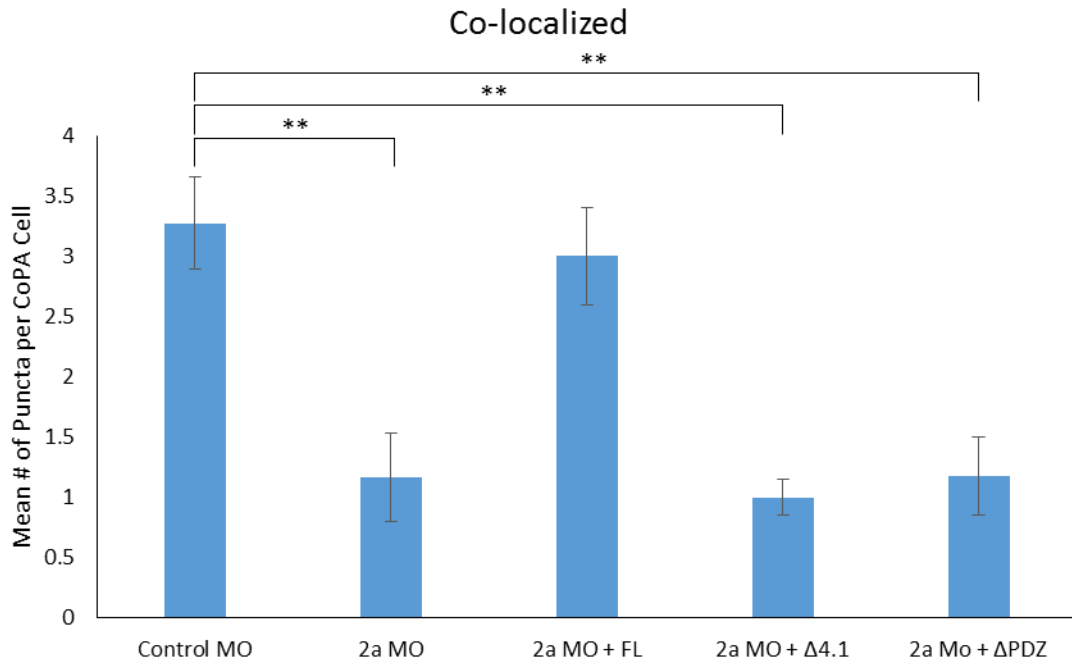


Figure 13. Analysis of colocalized synapsin1,2 and panMAGUK puncta. Bars indicate the number of colocalized puncta between RB axons and CoPA cell bodies (as determined by et101.2 GFP). Control MO, N = 11; 2a MO, N = 12; 2a MO + FL, N = 4; 2a MO + Δ4.1, N = 10; 2a MO + ΔPDZ, N = 11. ** indicates a p-value of <0.01. Bars indicate standard error.

SynCAM2a is Necessary for Proper Development of the Touch Response

Having characterized the molecular phenotypes through IF, all subsequent embryos were subjected to the touch response assay. **Figure 14** depicts characteristic responses for control MO-injected and 2a MO-injected embryos. Quantification of the responses showed that, on average, control MO-injected embryos failed to respond to the mechanical stimulus 3 percent of the time. Embryos injected with 2a MO failed to respond to the same stimulus 51 percent of the time, roughly a 17-fold increase in failure rate. Embryos coinjected with 2a MO and full-length *SynCAM2a* RNA showed no significant difference in failure rate when compared to control MO-injected fish. Coinjection of $\Delta 4.1$ or ΔPDZ RNA with 2a MO correlated with 13-fold and 15-fold increases in failure rate respectively (**Figure 15**). Ultimately, these findings show that *SynCAM2a* is required for efficacy of the touch response behavior, and that both the 4.1- and PDZ-binding motifs are essential for facilitating the actions of *SynCAM2a* in this circuit.

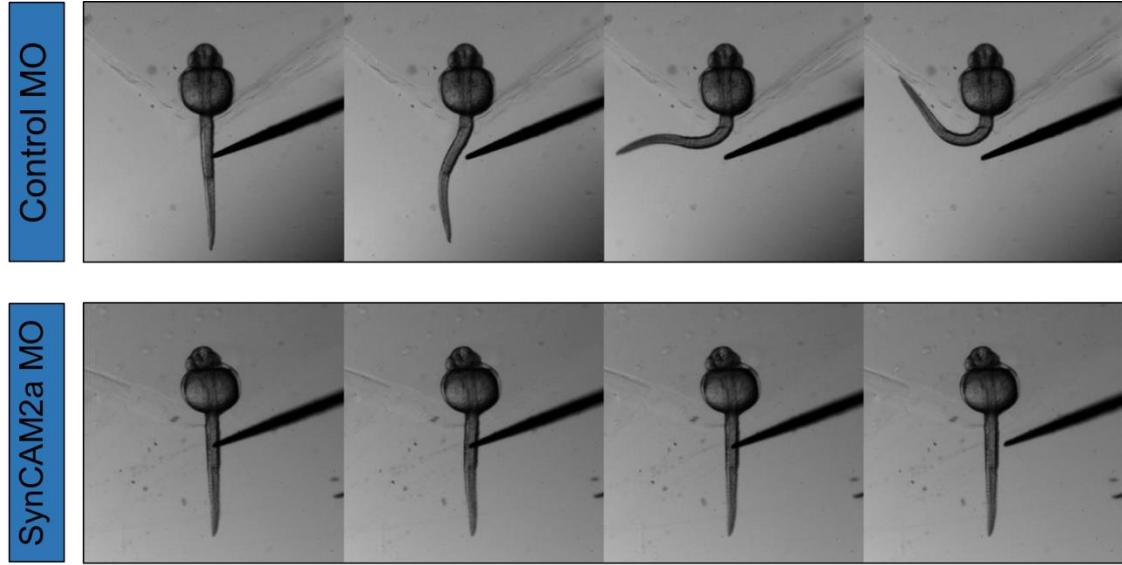


Figure 14. Characteristic responses to touch. Diagram illustrates a 40 ms timespan. Top panel shows successful response whereas bottom panel shows a failure to respond.

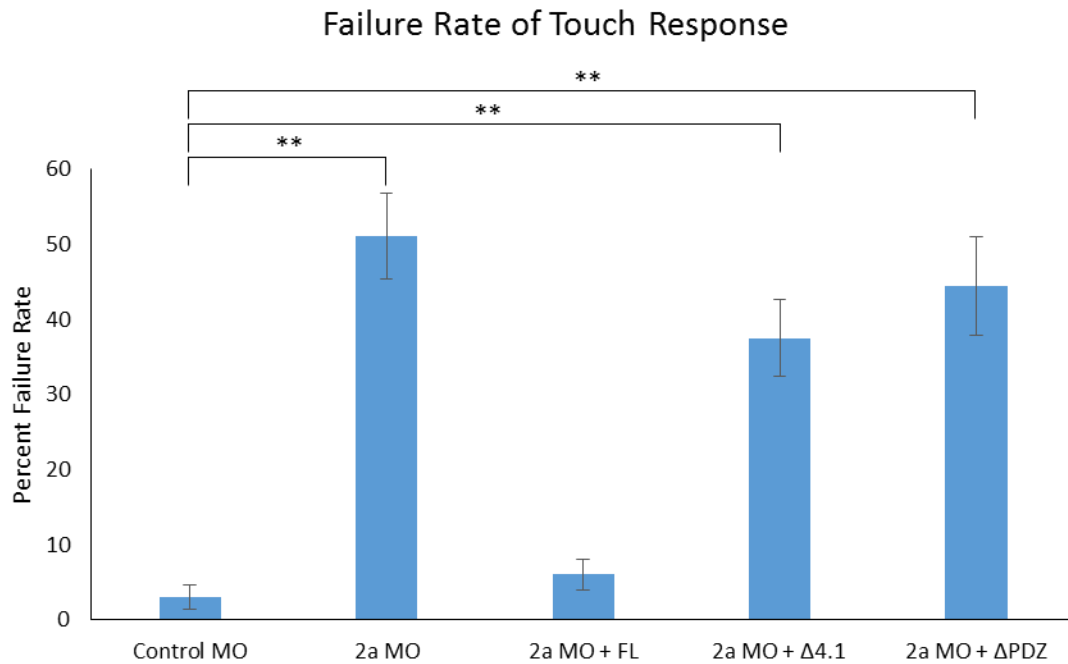


Figure 15. Analysis of embryonic response to touch. Bar height indicates the number of failed responses as a percentage of total times touched. Control MO, N = 20; 2a MO, N = 20; 2a MO + FL, N = 20; 2a MO + $\Delta 4.1$, N = 20; 2a MO + ΔPDZ , N = 20. ** = $p < 0.01$. Bars indicate standard error.

Discussion

Currently, the treatment and prevention of certain neurologic disorders is limited by an insufficient understanding of neurodevelopmental processes. In particular, no complete model has been established which details the complicated mechanisms by which a synapse develops. Being that disorders such as ASD, mental retardation and schizophrenia have been postulated to arise from synaptic dysfunction, the need for such a model is paramount. This project aimed to identify and characterize a key constituent involved in the process of synapse formation.

Recent studies have suggested that SynCAM family proteins are important for promoting and supporting synaptogenesis. Our study utilized *Danio rerio* to test this hypothesis and functionally characterize the zebrafish ortholog of human *SynCAM2*. Knowing that the zebrafish ortholog, *SynCAM2a*, is expressed in touch-sensitive RB sensory cells, this study was able to employ assays which considered the protein's function at both the molecular and behavioral levels.

Mechanisms of SynCAM2a in Synaptic Development

IF showed that knockdown of SynCAM2a results in decreased localization of several proteins to synaptic terminals. In the presynaptic terminal, loss of SynCAM2a led to decreased amounts of synapsin proteins 1 and 2 as well as synaptotagmin 2B. Decreased levels of synapsin proteins suggest that presynaptic terminals deficient in SynCAM2a are less able to cluster synaptic vesicles at active zones and are inefficient at recycling vesicles after synaptic transmission. This decreased ability of terminals to regulate their

reserve pools of synaptic vesicles would ultimately lead to diminished neurotransmitter release and a weakened synaptic signal. Decreased levels of synaptotagmin 2B would have a similar effect on synaptic strength. Synaptotagmin proteins are directly responsible for docking synaptic vesicles to the active zone in response to calcium influx. Lowered levels of synaptotagmin proteins would thus result in decreased release of neurotransmitter, and again, weakened synaptic strength.

In the postsynaptic terminal, SynCAM2a knockdown led to decreased PanMAGUK staining. This result suggests that postsynaptic scaffolding is not being formed properly in CoPA cells. As a consequence, it is likely that less neurotransmitter receptors are available at the postsynaptic membrane. In the absence of excitatory **AMPA receptors**, Na⁺ influx following presynaptic neurotransmitter release would be lessened. In turn, the postsynaptic cell would experience a smaller **postsynaptic potential**, an inability to achieve threshold, and ultimately, would fail to become activated.

In addition to affecting the efficacy of each terminal separately, SynCAM2a knockdown led to decreased colocalization of synapsin1,2 and PanMAGUK puncta. This result suggests that not only is synaptic transmission being weakened in existing synapses, but also, that the total number of synapses has decreased. In the context of the touch response circuit, the overall effect is that the stimulation of RB cells is less likely to result in activation of CoPA cells for SynCAM2a knockdown fish.

Formation of the Touch Response Neuronal Circuit via SynCAM2a

IF showed that neurotransmission presiding in the first synaptic junction of the touch response circuit is altered as a result of SynCAM2a knockdown. It would thus stand to reason that the behavior associated with said circuit would be affected as well. Our results suggest that this is indeed the case; SynCAM2a knockdown had a significant effect on the efficacy of the touch response. By lowering endogenous expression of SynCAM2a protein, synaptic strength at the initial neuronal junction of the circuit was weakened, and 2a MO-injected fish were less able to respond to the mechanical stimulus. Together, these results show that SynCAM2a is important for proper formation of synapses and is necessary for building complete neuronal circuits capable of functional behavior. Furthermore, our rescue experiments showed that both the 4.1- and PDZ-binding motifs are necessary to facilitate the actions of SynCAM2a as neither of the deletion constructs were able to rescue either the molecular or behavioral wild-type phenotypes.

Conclusions

Previous studies have shown SynCAM proteins to be important for promoting synapse assembly by serving as the ‘glue’ which keeps pre- and postsynaptic elements tightly bound together³⁸. Furthermore, mutations in SynCAM family proteins have been shown to decrease synaptic density *in vitro* and have been implicated in the pathogenesis of several neurodevelopmental diseases. This study demonstrates that SynCAM2a is important for facilitating proper synapse formation, and that without it, molecular phenotypes can manifest themselves into neuronal and behavioral anomalies. Ultimately,

this study reinforces the notion that disruptions in *SynCAM* genes could contribute to the onset of neurodevelopmental diseases such as ASD.

The results of this study may also provide insight into the redundancy and compensation mechanisms among SynCAM proteins. If *SynCAM2a* was fully responsible for initiating synaptogenesis at the RB:CoPA junction, one might expect knockdown of the gene to result in a complete loss of synapses. As was demonstrated in this study however, synapses still formed at the RB:CoPA junction. While the morpholino likely did not establish a 100 percent knockdown, it is possible that other cell adhesion molecules were compensating for the loss of SynCAM2a. Further studies will be necessary to distinguish between these possibilities.

Future Directions

In part, this study was able to show that SynCAM2a knockdown disrupted the localization of proteins to the pre- and postsynaptic terminals. It will therefore prove beneficial to next consider the trafficking of transport packets along the neuron in SynCAM2a knockdown fish. Doing so will help to identify other components missing from the synapse, and thus, other proteins that SynCAM2a is responsible for recruiting. Along the same line, it will be important to specifically look at postsynaptic neurotransmitter receptors to determine if their localization has been disrupted as well. Disruption of postsynaptic receptors would further confirm that synaptic strength decreases in the absence of SynCAM2a. Moreover, it will help to illustrate the notion that SynCAM2a is important for assembly of both the pre- and postsynaptic terminals.

Another experiment that we plan on performing involves the **misexpression** and **overexpression** of *SynCAM2a*. By increasing expression of *SynCAM2a* beyond endogenous levels, and in abnormal locations throughout the nervous system, we will be able to determine if *SynCAM2a* has a synaptogenic effect, that is, if it has the potential to increase synaptic density and/or strength beyond wild-type levels. Moreover, it will be interesting to determine the behavioral effects that such aberrant synapse formation would have.

Perhaps the most important experiment that still needs to be conducted however is confirming the knockdown of *SynCAM2a*. Ideally, we would like to perform a **western blot** to confirm that the morpholino is preventing translation of *SynCAM2a* RNA to protein. This experiment would also be important for confirming that the rescue constructs are being translated into appropriate amounts of protein, mimicking wild-type expression levels. In order to perform this experiment, we must first find an antibody that specifically recognizes zebrafish *SynCAM2a*.

Although we have not been able to confirm the knockdown through this ‘gold standard,’ our results are not to be discredited. The rescue experiments with full-length *SynCAM2a* RNA strongly suggest that the morpholino is working, and that the observed phenotypes are a result of lower *SynCAM2a* protein levels. One could imagine that if the morpholino was causing aberrant cell death, or disrupting the expression of other proteins, a similar phenotype to what was observed might be possible. If this were the case however, one would not expect coinjection of full-length *SynCAM2a* RNA to rescue the phenotype. As was demonstrated in these experiments however, full-length RNA was

indeed able to fully rescue the molecular and behavioral phenotypes. Consequently, it is safe to conclude that lowered levels of SynCAM2a protein, and not off-target effects, caused the phenotypes associated with morpholino injection.

In addition to confirming knockdown from morpholino injection, we also plan to repeat these experiments with a new *SynCAM2a* knockout line. Utilizing a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) protocol, we were able to generate fish with a complete knockout of *SynCAM2a*. With these fish, the complications associated with morpholino injection will be irrelevant, and any observed phenotypes will presumably be a direct result of *SynCAM2a* deficiency.

Taken together, these additional experiments will help further elucidate the function of SynCAM2a at the synapse. Moreover, they will provide a more complete understanding of how the loss of a single cell adhesion molecule can affect not only the formation of a synapse, but the mechanisms of a neuronal circuit, and ultimately, the behavior of an organism. Once the foundation of synaptic formation has been established, researchers will be ever closer to combatting the neurodevelopmental diseases which plague society today.

Appendix I: Glossary of Terms and Abbreviations (Alphabetical)

<i>Action potential:</i>	A rapid and transient electrical nerve impulse of about one millisecond that is initiated at the axon hillock. Action potentials are propagated down the length of the axon until they reach the presynaptic terminal, at which point the electrical impulse triggers the influx of Ca^{2+} and the release of neurotransmitters at the synapse ¹ .
<i>Active zone:</i>	The site of synaptic vesicle docking and neurotransmitter release.
<i>Antibody:</i>	Large proteins produced by the immune system to recognize and neutralize foreign objects such as bacteria or viruses ³⁹ .
<i>Antigen:</i>	A substance that triggers the production of antibodies by the immune system when introduced into the body. Antigens are usually foreign proteins from an organism other than the host ³⁹ .
<i>Assay:</i>	An analytical procedure used to qualitatively assess or quantitatively measure the presence, amount, or functional activity of a target entity.
<i>AMPA Receptor:</i>	Also known as the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor. AMPA receptors are glutamatergic receptors which mediate excitatory synaptic transmission in the central nervous system. See also: glutamatergic neurotransmission.

<i>Cell:</i>	The basic structural, functional and biological unit of all living organisms.
<i>Circumferential:</i>	The superlative of circumference. Used to describe information pertaining to the outermost edge of a surface or along the boundary of an object.
<i>Contralateral:</i>	Denotes the side of the body opposite to that of a particular structure or condition. See also: ipsilateral.
<i>Depolarization:</i>	A change in voltage across a cell's membrane which renders the membrane potential more positive, or less negative. In some cells, a large enough depolarization can lead to the onset of an action potential. See also: voltage.
<i>Epitope:</i>	The specific part of an antigen that is recognized by an antibody. See also: antibody.
<i>Embryo:</i>	A developmental stage relating to multicellular diploid eukaryotes in the earliest stage of development. Characterized by the time of first cell division until birth, hatching, or germination.
<i>Tissue Fixation (fixed):</i>	A chemical process by which biological tissues are preserved from decay, thereby terminating any ongoing biochemical reactions.
<i>Ganglion:</i>	A mass of nerve cell bodies. Plural: ganglia.

<i>Gap junction:</i>	A small tubular protein structure which allows for the movement of ions from one cell's interior to the next. The common form of an electrical synapse.
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<i>Gene:</i>	A hereditary portion of DNA and/or RNA within a living organism that is responsible for encoding functional products, such as a protein or functional ribonucleic acid.
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<i>Glial cell:</i>	A non-neuronal cell which provides support and protection for neurons within the central nervous system. See also: Schwann cell.
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<i>Glutamatergic neurotransmission:</i>	Synaptic transmission via the neurotransmitter glutamate. Synapses that employ glutamatergic transmission (called glutamatergic synapses) can involve different types of receptors that bind glutamate and other amino acid derivatives that mimic the action of glutamate ¹ . See also: neurotransmitter.
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<i>Heterophilic binding:</i>	A type of cell-cell adhesion interaction that involves a cell adhesion molecule on one cell directly binding to a different cell adhesion molecule on an adjacent cell ⁴⁰ .
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<i>Homophilic binding:</i>	A type of cell-cell adhesion interaction that involves a cell adhesion molecule on one cell directly binding to the same kind of cell adhesion molecule on an adjacent cell ⁴⁰ .
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<i>Immunofluorescent staining (IF):</i>	A technique used in molecular biology to detect specific antigens—typically proteins—in biologic tissues by means of fluorescently-tagged antibodies. See also: antibody.
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<i>Immunoglobulin (Ig):</i>	A protein domain which aids in the recognition, binding, or adhesion processes of a cell. See also: protein.
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<i>Ipsilateral:</i>	Belonging to the same side of the body. See also: contralateral.
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<i>Knock Down:</i>	An experimental technique in which the expression of one or more of an organism's genes is reduced. See also: gene, knock out.
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<i>Knock Out:</i>	An experimental technique in which one or more of an organism's genes are made inoperative. See also: gene, knock down.
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<i>Misexpression:</i>	The expression of a gene product (RNA or protein) at a time or place different from that of wild-type expression. See also: gene, overexpression.
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<i>Model organism:</i>	A non-human species, usually having significant orthology with humans, that is studied to understand particular biological phenomena. Model organisms are often employed when human experimentation would be logistically or ethically impossible. See also: ortholog.
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<i>Morpholino:</i>	A molecule used to modify gene expression. Translation-blocking morpholinos work by binding anti-sense to mRNA and

	preventing the cell's translational machinery from making functional protein.
<i>Mutagenesis:</i>	A process by which the genetic information of an organism is changed, resulting in a mutation. See also: gene.
<i>Mutant:</i>	Used to describe either an organism harboring base-pair sequence changes within the DNA of its genome, or the altered gene itself. See also: gene.
<i>Neuron:</i>	An electrically excitable cell within the nervous system that transmits information either through electrical or chemical signals.
<i>Neurotransmitter:</i>	A chemical used to transmit information across a synapse from one neuron to another target neuron.
<i>Ortholog:</i>	Genes in different species that originated from a single gene in a common ancestor ⁴⁰ . See also: gene.
<i>Overexpression:</i>	The expression of a gene product (RNA or protein) in an amount greater than wild-type expression. See also: gene, misexpression.
<i>Phenotype:</i>	An organism's observable characteristics, such as morphology, development, physiological properties, and behavior.
<i>Photobleaching:</i>	The photochemical destruction of a dye or fluorophore resulting from excess exposure to light. Photobleaching is a common problem in immunofluorescent staining which complicates the detection of antigens.

<i>Postsynaptic Potential:</i>	The voltage change experienced by a dendrite and/or soma of a postsynaptic cell in response to synaptic transmission.
<i>Protein:</i>	A biochemical macromolecule. Proteins are comprised of amino-acid polymers folded into globular and fibrous forms. Active proteins perform enzymatic catalysis and assist biological reactions.
<i>Punctum:</i>	A small distinct point. In immunofluorescent experiments, puncta (plural) represent the location(s) of an antigen(s). See also: antigen, immunofluorescent staining.
<i>Reductionism:</i>	A philosophical belief that maintains a complex system can be subdivided, or reduced, into individual constituents.
<i>Schwann cell:</i>	The principal glia of the peripheral nervous system. See also: glial cell.
<i>Synapse:</i>	The structure that permits a neuron to communicate with another cell, neural or otherwise, via electrical or chemical signaling.
<i>Synaptogenesis:</i>	The formation of synapses between neurons in the nervous system. See also: synapse.
<i>Tetrapod:</i>	The phylogenic superclass which comprises the first four-limbed vertebrates and their descendants, including amphibians, reptiles, birds, and mammals.
<i>Transduction:</i>	The process by which a signal is converted from one form to another. At a chemical synapse, the electrical signal of an action

	potential gets transduced into a chemical signal in the form of neurotransmitter. See also: action potential, synapse.
<i>Transgenesis:</i>	The process of introducing an exogenous, or foreign, gene into a living organism. See also: gene.
<i>Vertebrate:</i>	The subphylum comprised of organisms which are built along the basic chordate body plan: a stiff rod running through the length of the animal (vertebral column or notochord).
<i>Voltage:</i>	A difference in electrical potential, measured in units of volts or joules per coulomb. Voltages can be established across biologic membranes through the action of ion pumps and channels.
<i>Western Blot:</i>	A common analytical technique which utilizes antibody-binding to identify the presence of specific proteins in a tissue sample. See also: antibody.
<i>Wild-type:</i>	The phenotype normally found in nature for a specific species. See also: phenotype.
<i>Z-stack:</i>	The three-dimensional composite of two or more planar images.

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