Investigating the role of Wnt signaling during retinotectal circuit development

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List of Abbreviations

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
APC	adenomatous polyposis coli
APV	(2R)-amino-5-phosphonopentanoic acid
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
CaMKII	calcium/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CBP	creb-binding protein
Cdc42	cell-division control protein 42
CE	convergent extension
cGMP	cyclic guanosine monophosphate
ChR	channel rhodopsin
CK1ε	casein kinase 1ε
CNS	central nervous system
CPP	3-(2-Carboxypiperazin-4-yl) propyl-1-phosphonic acid
CRD	cysteine rich domain
CSC	compound synaptic current
DAG	diacylglycerol
DRG	dorsal root ganglion
Dsh/Dvl	dishevelled
D-V	dorsal-ventral (axis)
EE	enriched environment
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
Evi	evenness interrupted
Fz/Fzd	frizzled
GABA	gamma-aminobutyric acid
GFP	green fluorescent protein
GPI	glycosyl phosphatidylinositol
GSK3b	glycogen synthase kinase 3β
GTP	guanosine triphosphate
HCG	human chorionic gonadotropin
HFS	high-frequency stimulation
IP3	inositol triphosphate
JNK	c-jun N-terminal kinase
LFS	low-frequency stimulation
LGN	lateral geniculate nucleus
LRP	lipoprotein receptor-like protein
LTD	long-term depression

LTP	long-term potentiation
MAP	microtubule-associated protein
MBSH	modified Barth's solution
MEPP	miniature endplate potential
mEPSC	miniature excitatory postsynaptic current
MK-801	dizocilpine
M-L	medial-lateral (axis)
mPSC	miniature postsynaptic current
mRNA	messenger ribonucleic acid
MS-222	tricaine mesylate
MT	microtubule
N	synapse number
nAChR	nicotinic acetylcholine receptor
NFAT	nuclear factor of activated T-cells
NMDAR	N-methyl-D-aspartate type glutamate receptor
NMJ	neuromuscular junction
NO	nitrous oxide
NOS	nitrous oxide synthase
N-T	nasal-temporal (axis)
OT	optic tectum
PBS	phosphate buffer solution
PCP	planar cell polarity
PDE	phosphodiesterase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMSG	pregnant mare's serum gonadotropin
PNS	peripheral nervous system
PPR	paired-pulse ratio
Pr	probability of release
PSD	postsynaptic density
PSD-95	postsynaptic density protein 95
PTX	picrotoxin
Q	quantal amplitude
R-C	rostral-cadual (axis)
RF	receptive field
RFP	red fluorescent protein
RGC	retinal ganglion cell
RM	repreated measure
RoR	tyrosine kinase-like orphan receptor
RTK	receptor tyrosine kinase
Ryk	receptor-like tyrosine kinase
SC	superior colliculus
SEM	standard error of the means

sEPSC	spontaneous excitatory postsynpatic current
SFK	src-family kinases
sFRP	soluble frizzled-related protein
sPSC	spontaneous postsynaptic current
STDP	spike-timing dependent plasticity
STP	short-term plasticity
STVE	short-term enhanced visual experience
TCF/LEF	T-cell factor/ lymphoid-enhancer factor
TDBL	total dendritic branch length
TDBN	total dendritic branch number
TenT	tetanus toxin
tLTD	timing long-term depression
tLTP	timing long-term potentiation
TRP	transient receptor potential
TTX	tetrodotoxin
WT	wild-type

Abstract

The development of topographic maps in the visual system requires both genetic and sensory experience-dependent factors, but how these different mechanisms interact is poorly understood. In the retinotectal system, Wnt3A is a target-derived ligand that influences axon guidance and receptive field plasticity, suggesting that it could facilitate circuit development through both experience-dependent and experience-independent pathways. In the present study, we aimed to clarify the functional role of Wnt signaling in the developing retinotectal circuit of *Xenopus laevis* tadpoles. We first used a transgenic reporter line (pbin7Lef-dEGFP) for canonical Wnt signaling to confirm the presence of Wnt activity in the optic tectum during retinotopic refinement. We found that Wnt3A expression in postsynaptic tectal neurons increases miniature excitatory postsynaptic current (mEPSC) frequency, AMPA/NMDA ratios, and the density of postsynaptic puncta, indicating a role for Wnt3A in promoting synaptic maturation. Overexpression of Wnt3A in tectal neurons also increased total dendritic branch length after an 8-hour imaging period. Moreover, subjecting animals to visual stimulation, but not darkness, increased the length of dendritic branches in Wnt3A-expressing neurons relative to controls, suggesting that Wnt3A may promote dendritic branch growth through a sensory-dependent mechanism. We also investigated the influence of Wnt signaling in regulating retinal ganglion cell (RGC) axon morphology, showing that the disruption of presynaptic Wnt signalling increases the number of axon branches. Together, these results demonstrate that Wnt signaling has diverse roles at both sides of the synapse during retinotectal remodeling, highlighting the versatility of these pathways in coordinating circuit development.

Résumé

Le développement de cartes topographiques dans le système visuel nécessite à la fois des facteurs génétiques et sensorio-dépendants de l'expérience, mais la façon dont ces différents mécanismes interagissent est peu comprise. Dans le système rétinotectal, Wnt3A est un ligand qui influence le guidage axonal et la plasticité du champ réceptif, ce qui suggère qu'il pourrait faciliter le développement du circuit par des voies dépendantes et indépendantes de l'expérience. Dans l'étude présente, nous avons cherché à clarifier le rôle fonctionnel de la signalisation Wnt dans le circuit rétinotectal en développement des têtards Xenopus laevis. Nous avons d'abord utilisé une lignée rapporteur transgénique (pbin7Lef-dEGFP) pour la signalisation canonique Wnt afin de confirmer la présence d'activité Wnt dans le tectum optique lors du raffinement rétinotopique. Nous avons constaté que l'expression de Wnt3A dans les neurones tectaux postsynaptiques augmente la fréquence des courants postsynaptique excitateur miniature (mEPSC), les rapports AMPA/NMDA et la densité des points ponctuels postsynaptiques, ce qui indique un rôle de Wnt3A dans la promotion de la maturation synaptique. La surexpression de Wnt3A dans les neurones tectaux a également augmenté la longueur totale des branches dendritiques après une période d'imagerie de 8 heures. De plus, le fait de soumettre les animaux à une stimulation visuelle, mais pas à l'obscurité, a augmenté la longueur des branches dendritiques dans les neurones exprimant Wnt3A par rapport aux témoins, ce qui suggère que Wnt3A peut favoriser la croissance des branches dendritiques par un mécanisme sensorio-dépendant. Nous avons également étudié l'influence de la signalisation Wnt dans la régulation de la morphologie des axones des cellules ganglionnaires rétiniennes (RGC), montrant que la perturbation de la signalisation présynaptique Wnt augmente le nombre de branches axonales. Ensemble, ces résultats démontrent que la signalisation Wnt joue divers rôles des deux côtés de

la synapse lors du remodelage rétinotectal, soulignant la polyvalence de ces voies dans la coordination du développement du circuit.

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Author Contributions

Chapter 1 – Literature review: This chapter was written by me with revisions from Dr. Edward Ruthazer. Section 1.3 is modified from a review article in which I was the second author called "Approaches and Limitations in the Investigation of Synaptic Transmission and Plasticity", Frontiers in Synaptic Neuroscience, 24 July 2019. The sections adapted from this article were written by me, with major edits provided by Dr. Edward Ruthazer and Dr. Stephen Glasgow. Figure 1.1 is adapted from Feldheim & O'Leary, 2010 (Cold Spring Harbor Laboratory Press). Figure 1.2 is adapted from Kutsarova et al., 2016 (Frontiers in Neural Circuits, Creative Commons CC-BY license). Figure 1.4 was reused from Mulligan & Cheyette, 2016, (S. Karger AG, Basel).

Chapter 2 - Methodology: I wrote this chapter with revisions provided by Dr. Edward Ruthazer.

Chapter 3 - Results: Dr. Edward Ruthazer and I planned all the experiments from this section. I carried out all the experiments and data analysis. Anne Schohl designed and produced the BICS2-XWnt3A-GFP (section 3.2), BICS2-XWnt3A-mCherry (section 3.3) and pCS-Wnt3A (section 3.4) constructs used in this research. Section 3.6 was done in collaboration with Virginie Chotard, who helped me with the collection and analysis of the data and produced the time-series image in Figure 3.6A.

Chapter 4 - Discussion: I wrote this chapter with revisions provided by Dr. Edward Ruthazer. Figure 4.1 was made by me, but its design was inspired by Teo & Salinas (2021).

Preface

It is perhaps my own bias that I find the most fascinating questions to also be the most timeless. Before the advent of scientific methodologies, it was philosophy that sought to provide answers to fundamental questions about the natural world. The question of how an organism develops into a mature form is nearly as old as philosophy itself, being first posed and studied by Aristotle. His keen observation that the morphological structure of an organism was so remarkably suited to its function spurred a curiosity to understand the enigmatic processes through which development occurs. Ontogeny, as it was known back then (derived from the Greek "origin of being"), sought to characterize the series of morphological and behavioral changes that occur throughout the lifespan of an individual organism and the degree to which behavioral repertoires can be altered through learning.

It is from the inchoate natural philosophy of Aristotle that modern science has evolved, and with it, a greater understanding of the remarkably complex and dynamic process of development. Contributing to our enhanced understanding of biological development has been the more recent emphasis on characterizing the developmental mechanisms of biology's greatest exemplar of adaptive plasticity: the brain. With its billions of neurons, making trillions of synapses that are continuously rearranged and refined, the brain and the neural circuits from which it's comprised enables a nearly inexhaustible capacity for learning. Elucidating the intricate mechanisms that give rise to an organ of such staggering complexity poses a monumental challenge for developmental biology. However, with this challenge we are presented with an unprecedented opportunity to understand the limits of our capacity to learn, grow and evolve.

The scope of this thesis aims to characterize the molecular events that govern the specification of synaptic connections in the developing nervous system, as well as those events that make the brain so uniquely receptive to sensory information filtered through the external world. In particular, I focus on the contribution of the intercellular signaling molecules, Wnts, in guiding the development of the vertebrate visual system. Just as the visual system functions as the nexus between the internal and external world, so too are Wnts poised at the nexus of regulating hardwired synaptic connectivity and activity-dependent plasticity, making them prime targets for uncovering the mechanisms by which brains are built.

Chapter 1: Literature Review

1.1 The Developing Retinotectal Circuit

1.1.1 *Xenopus laevis* as a model for understanding neural circuit development

Our ability to generate fundamental insights into the mechanistic principles underlying neural circuit development crucially depends on the model systems and methodologies by which we direct our scientific inquiries. For many decades, animal models such as the African claw-toed frog, *Xenopus laevis*, and zebrafish (*Danio rerio*) have provided scientists with an ideal opportunity to study the enigmatic process of neural circuit development. In particular, the retinotectal system of *Xenopus laevis* has generated many important insights into how neural circuits are wired due to various features of this model system that render it uniquely suitable for addressing these types of questions. The most obvious advantage of studying developmental processes in *Xenopus laevis* is that their eggs are both large--making them amenable to experimental manipulation--and develop externally, which facilitates the study of neural circuits in their earliest stages of development (as opposed to mammalian models which develop *in-utero*). Albino strains of *Xenopus laevis* are especially useful since these animals are mostly translucent in larval stages, enabling *in vivo* high-resolution imaging of actively growing neurons. Moreover, *Xenopus laevis* tadpoles develop a functional visual system in less than a week that allows them to perform visually-guided behaviors such as predator avoidance. Together, these attributes facilitate the investigation of structural and functional aspects of visual circuits.

The retinotectal circuit of *Xenopus laevis* is not just a practical system for studying neurodevelopment, but an eminently fascinating one given its complexity and highly precise structural arrangement. The retinotectal system is one such example of a topographically organized circuit whereby neighboring afferents from the retina project to neighboring target neurons in the optic tectum (OT), which preserves the spatial order of visual inputs. The high degree of topographic precision is especially impressive given how far retinal ganglion cell (RGC) afferents must travel before finding their appropriate targets in the contralateral OT. However, the mechanisms responsible for this specified organization of synaptic connections remain to be fully elucidated.

1.1.2 A tale of two hypotheses: nature vs. nurture in the developing brain

At a high level, the development of a neural circuit presupposes an ability to integrate and respond to information arising from two possible sources. The first is information that is intrinsic to the organism itself, which resides in genetic programs that act as a hardwired blueprint for generating an animal of a given species. The second is extrinsic cues coming from an organism's environment that permit it to adaptively respond to changing contexts. Both sources of information are crucial for the development of sensory circuits, as they must be rigid enough to retain general functions but malleable enough to tune into specific environmental conditions. The visual system accomplishes this by making use of genetic information that specifies synaptic matching by utilizing the differential expression of cell surface molecules, and environmental information that translates sensory stimuli into neuronal firing patterns to distinguish appropriate synaptic connectivity. Significant advances have been made in understanding the relative

contributions of genetic and activity-dependent mechanisms in nervous system development, due in large part to studies in the vertebrate visual system.

The importance of genetic determinants in establishing synaptic connectivity was convincingly demonstrated by Roger Sperry who showed in newts and frogs that inducing axon regeneration following surgical rotation of the eye by 180 degrees did not interfere with the topographic projection of RGCs to their normal target regions within the OT (Sperry, 1943). These observations led him to propose the "chemoaffinity hypothesis," postulating that presynaptic and postsynaptic associations are specified by the differential expression of cytochemical tags (Meyer & Sperry, 1976; Sperry, 1963). The graded expression of such tags in target and afferent neurons could uniquely identify the position of synaptic partners to facilitate topographic mapping. Sperry's hypothesis was validated with the discovery and characterisation of Ephrins as retinotopic mapping molecules (Flanagan & Vanderhaeghen, 1998; O'Leary & Wilkinson, 1999), however it is unlikely that this mechanism is sufficient to establish the degree of precision required for topographic mapping and cannot account for the plasticity of neural circuits (Katz & Shatz, 1996).

Alternatively, an activity-dependent model for synaptic matching was put forth by Donald Hebb (Hebb, 1949), who proposed that correlated firing between presynaptic and postsynaptic neurons could selectively stabilize these connections. His hypothesis was later expanded upon to include a mechanism by which uncorrelated firing results in synaptic weakening or elimination (Stent, 1973). Perhaps the most striking example of activity-dependent circuit development comes from the classic three-eyed frog experiments (Constantine-Paton & Law, 1978), where eye primordium was transplanted and integrated as a functional eye, resulting in eye-specific segregation of RGC afferents from two eyes into ocular dominance bands. Since

retinal afferents in frogs normally innervate only the contralateral tectum, there should be no genetically encoded mechanism to distinguish RGC afferents from different eyes, thus the inter-tectal segregation of RGC inputs would have to be caused by differential activity patterns. Consistent with this idea, blocking activity in the optic nerve of the supernumerary eye prevents the segregation of inputs into ocular dominance bands (Reh & Constantine-Paton, 1985).

Although it may appear as though hardwired and activity-dependent mechanisms are distinct processes, accruing evidence suggests that they not only act in concert with one another, but are inextricably linked and interdependent (Cline, 2003). The following section will provide an overview of how these mechanistic events play out over the course of retinotectal development.

1.1.3 The process of retinotectal circuit development

In the vertebrate visual system, RGCs constitute the sole projections from the eye that relay visual information to the brain. In fish and frogs, there are at least 10 distinct tectal and pretectal arborization regions, with the most extensive projections terminating in the OT (Easter & Taylor, 1989; Lazar, 1973). The OT in amphibians is analogous to the superior colliculus (SC) in mammals (Butler & Hodos, 2005; Schiller, 1972), both of which receive topographically oriented projections that direct visually guided movements and behaviors (Cang & Feldheim, 2013). In *Xenopus laevis*, all RGC projections destined for the OT cross the optic chiasm to innervate the contralateral hemisphere. The topographic orientation of these projections can be represented along orthogonal axes, with the nasal-temporal (N-T) axis of RGC somata in the eye terminating along the rostral-caudal (R-C) axis (equivalent to the anterior-posterior axis) of the

tectum, and dorsal-ventral (D-V) RGCs projecting along the medial-lateral (M-L) axis of the tectum. This high-fidelity mapping of visual space in the tectum enables the execution of coordinated motor commands via recipient tectal neurons (Cang & Feldheim, 2013; Liu et al., 2016). Thus, the topographic structure of the retinotectal circuit contributes to its proper functioning.

The development of this retinotopic map begins as soon as RGC axons innervate the tectum around stage 39 (Nieuwkoop & Faber, 1994). In this early stage of development, there are numerous molecular gradients in the eye and tectum that act in concert to generate a crude topographic circuit (McLaughlin & Leary, 2005) capable of driving visually mediated responses in postsynaptic tectal neurons (Holt & Harris, 1983). These nascent RGC projections initially display a simple morphological structure, but upon termination in their appropriate tectal regions undergo a process of extensive arbor expansion and elaboration (Fraser & O'Rourke, 1990; Sakaguchi & Murphey, 1985). This transient phase of dynamic arbor expansion functions as a putative strategy by which RGC axons explore their target area in search of suitable synaptic partners. Synapses formed during this period are labile but can be selectively strengthened and stabilized via Hebbian mechanisms that detect the coincident activation of adjacent synapses (Kutsarova et al., 2016). And since neighboring RGCs in the retina are more likely to have correlated activity patterns due to their spatial proximity, tectal neurons can use this input to preferentially stabilize topographically ordered connections.

While these dynamic morphological rearrangements are taking place, functional synaptic properties also undergo a gradual tuning. The number of inputs onto tectal neurons increases substantially between stages 42-48 as indicated by a steady rise in the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) over this period (Pratt & Aizenman, 2007). The

increase in synaptic inputs can be attributed to the formation of new synaptic contacts as well as the "unsilencing" of synapses containing only N-methyl-D-aspartate type glutamate receptors (NMDARs) via the recruitment of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) (Isaac et al., 1995; Liao et al., 1995). Correspondingly, the ratio of AMPAR to NMDAR currents increases gradually over the course of development and reflects a maturation of nascent synapses (Wu et al., 1996). This increase in synaptic drive due to the addition of new synapses is partially offset by a decrease in sEPSC amplitude and a transient increase in neural excitability during initial synapse formation that declines shortly after, suggesting homeostatic mechanisms regulate synaptic input/output during development to maintain it at a stable level (Pratt & Aizenman, 2007). The excitatory/inhibitory balance of synaptic connections is also modulated, as gamma-aminobutyric acid (GABA) inputs shift from inducing depolarizing currents early in development to hyperpolarizing currents in later development (Akerman & Cline, 2006). The temporal characteristics of neuronal firing patterns are also refined, as recurrent activity patterns become more temporally aligned and precise (Pratt et al., 2008).

This morphological and physiological refinement of retinotopic connections results in RGC arbors that are more spatially restricted within their target field such that the receptive field (RF) size of tectal neurons shrinks to become more topographically precise (Dong et al., 2009; Tao & Poo, 2005). By stage 48/49—about 7 days after RGCs first arrive in the tectum—the most dynamic phase of retinotectal remodelling abates to instantiate a more functionally mature visual circuit. However, the refinement of these synaptic connections is a continuous process, as the growing brain and eye of the tadpole must accommodate the addition of new tectal neurons and RGCs, respectively. In the retina, RGCs are added in a concentric manner, whereas in the tectum

cell proliferation takes place in the medial-caudal region (Gaze, 1972). Therefore, the maintenance of topographic connectivity requires a continual shifting of synapses—both nascent and mature—that preserves the relative spatial relations of these inputs.

This scheme of retinotopic development can be conceptually separated into three approximate and overlapping phases: topographic termination, arbor elaboration, and arbor refinement. In the first stage, hardwired molecular gradients dictate the topographic arrangement of RGC inputs. However, in the subsequent phases of retinotopic development, visually mediated activity patterns become increasingly important for refining topographic connectivity (Debski & Cline, 2002; Kutsarova et al., 2016; Ruthazer & Aizenman, 2010). The following sections will detail both the hardwired and sensory-dependent mechanisms that instruct retinotopic development as well as the interplay between the two.

1.1.4 Hardwired mechanisms for topographic patterning

In the retinotectal system, the Eph family of receptor tyrosine kinases (RTKs) and their complementary ligands, ephrins, play a crucial role in the establishment of the retinotopic map. Ephs and ephrins are divided into two subclasses, A and B, based on their homology and their receptor-ligand binding interactions (Kullander & Klein, 2002). Ephs and ephrin-Bs are transmembrane proteins, whereas ephrin-As are glycosyl phosphatidylinositol (GPI)-linked proteins. Ephrin signaling can be both bifunctional, attracting or repelling axons, as well as bidirectional, initiating signaling cascades through receptors and ligands on both sides of the synapse (Egea & Klein, 2007; Leary & McLaughlin, 2005).

EphAs and ephrin-As are the predominant cues for the mapping of nasal-temporal (N-T) RGC axons along the rostral-caudal (R-C) axis of the OT. In the retina, EphA receptors are expressed in a high-to-low T-N gradient, with a complementary gradient of ephrin-As in the OT that increases along the R-C axis (McLaughlin & Leary, 2005). Receptor-ligand coupling initiates axonal repulsion, which facilitates the mapping of temporal RGC axons, where EphA levels are high, to the rostral region of the tectum, where ephrin-A levels are low, and vice-versa (Hornberger et al., 1999). Although there are several mechanistic models that could explain how EphAs and ephrin-As mediate mapping along the R-C axis, perhaps the most convincing is a model whereby RGC axons compete to occupy space in the tectum according to where the repulsive forces are minimized (Triplett, 2014). A clever experiment relying on the ectopic expression of EphA3 in a subset of uniformly distributed islet2-RGCs, showed that two overlapping maps were generated with Islet2+ RGC axons occupying the rostral SC and wild-type (WT) RGC axons occupying the caudal SC (Brown et al., 2000). The formation of these two maps indicates that it is relative, rather than absolute, levels of EphA repulsive forces that determine the spatial positioning of RGCs, competing amongst themselves for termination zones in the SC (Reber et al., 2004). Furthermore, knocking down EphA5 in temporal RGCs (where expression is usually high), causes not only a caudal shift of these axons, but a concomitant rostral shift of WT nasal RGC axons that get displaced from their normal termination zones (Feldheim et al., 2004). Finally, Math5 mutant mice which retain only 5-10% of the normal number of RGCs show a degradation of their topographic order in the SC, presumably because of limited inter-axon competition (Triplett et al., 2011). Together, these studies demonstrate that EphA/ephrin-A signaling is a primary determinant for retinotopic mapping along the R-C axis by

facilitating competitive interactions between neighboring axons to take up space within SC/OT target zones.

Analogous to how EphA/ephrin-A gradients mediate retinotopic mapping along the R-C axis, EphBs and their ligands, ephrin-Bs, contribute to the mapping of dorsal-ventral (D-V) RGC axons to the medial-lateral (M-L) axis of the OT. In the retina, ephrin-Bs are expressed in a D-V decreasing gradient, with EphBs expressed in a M-L increasing gradient in the OT (Mann et al., 2002). Unlike EphA/ephrin-A signaling, EphB/ephrin-B signaling is attractive rather than repulsive, with signaling occurring in both forward (via the EphB receptor) and reverse (via the ephrin-B ligand) directions (McLaughlin et al., 2003). However, it is unlikely that EphB/ephrin-B signaling is the only factor for M-L mapping, since triple mutant mice for EphB1, B2 and B3 do not display a complete absence of topography along the M-L axis (Hindges et al., 2002), suggesting there may be additional mechanisms at play. Another molecular family that may contribute to M-L topography are Wnts. In the OT/SC of chicks/mice, Wnt3 is expressed in a high-to-low M-L gradient with receptor-like tyrosine kinase (Ryk) receptors expressed in a D-V decreasing gradient (Schmitt et al., 2006). Activation of Ryk by Wnt3 promotes axonal repulsion, while signaling through Frizzled (Fz/Fzd) receptors, which are evenly distributed across the retina, are shown to mediate chemoattraction *in vitro*. This evidence seems to suggest that there can be independent, but complementary signaling pathways that act in concert to establish mapping along the M-L axis of the tectum.

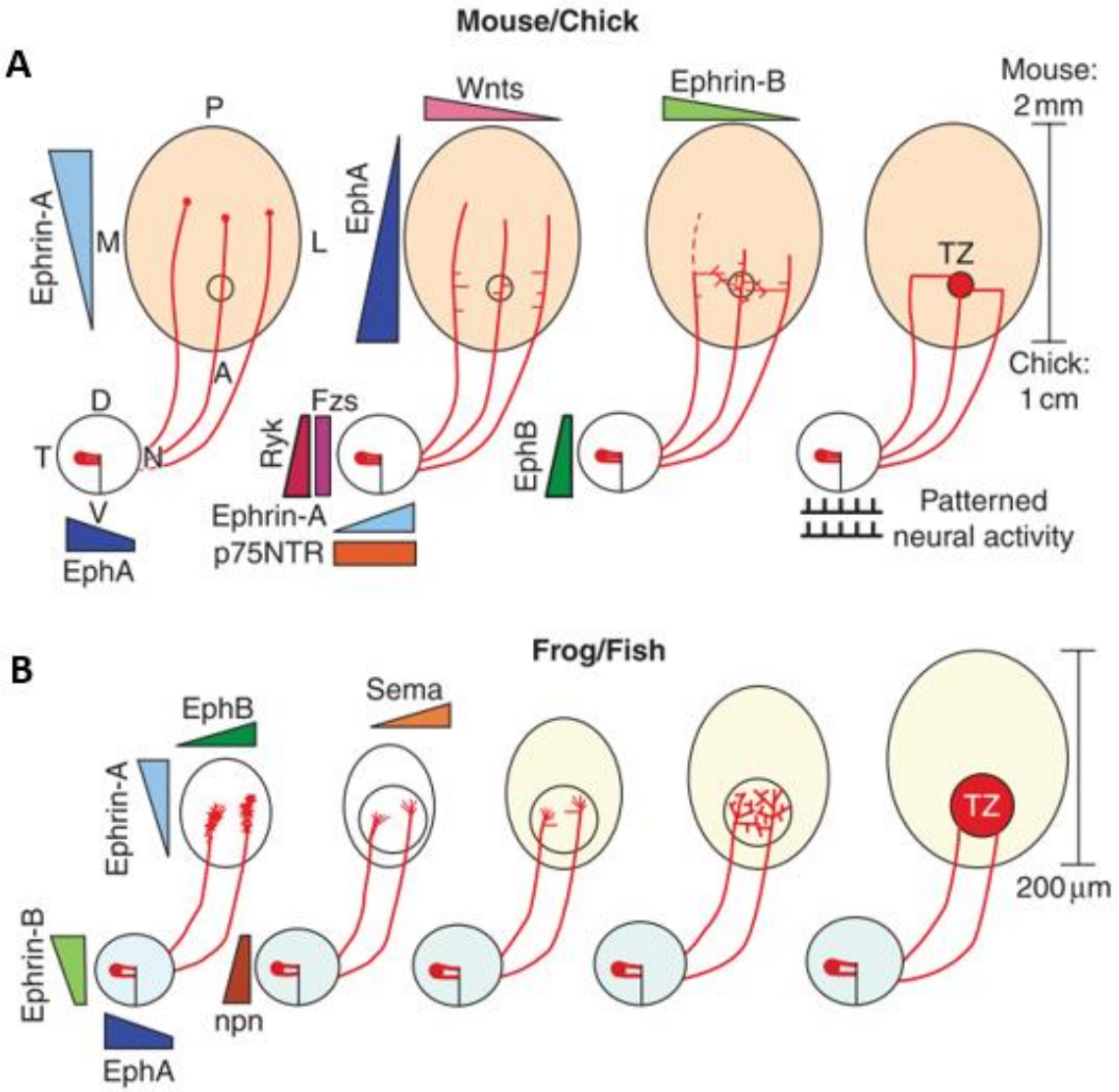

Figure 1.1: Chemotropic gradients establish topographic mapping of retinotectal/retinocollicular circuits. (A) In mice and chicks, EphA/ephrin-A gradients in the retina and SC/OT facilitate the mapping of N-T RGCs along the A-P axis of the SC/OT. Conversely, D-V EphB and Ryk gradients in the retina direct mapping along the M-L axis of the SC/OT. Patterned activity further sculpts retinotectal/retinocollicular afferents to ensure they innervate their appropriate target zone (TZ). (B) Similar chemotropic gradients are found in the retinotectal system of fish and frogs, performing an analogous function of mapping the N-T and D-V axes along the A-P (R-C) and M-L (D-V) axes of the OT. **(**Adapted from Feldhiem and O'Leary, 2010)

Moreover, there is accruing evidence that hardwired EphB and Wnt3 gradients function beyond mere axon guidance cues but can also contribute to the activity-dependent stabilization of topographically precise synapses at later stages of retinotopic development. In *Xenopus laevis*

tadpoles, ephrin-B1 reverse signaling in RGC axons appears to promote presynaptic stabilization and maturation without a significant alteration to arbor growth (Lim et al., 2008). The EphB/ephrin-B mediated stabilization of synapses is shown to proceed in two distinct phases, with reverse ephrin-B signaling first enhancing presynaptic release efficacy, followed by a postsynaptic recruitment of AMPARs in an NMDAR-dependent manner. Building on this work, Lim and colleagues (2010) investigated the involvement of both EphB and Wnt signaling in region-specific receptive field (RF) plasticity. They showed that RF shifts induced by a visual conditioning stimulus were disrupted by acutely inhibiting either of these signaling pathways. Since Wnt3A is highly expressed in the dorsal (medial) region of the OT, blockade of Wnt signaling reduced RF shifts in this region, whereas inhibiting EphB/ephrin-B signaling reduced RF shifts in the ventral tectum where EphB expression is highest. Because tectal RF shifts occur through an activity-dependent process (Engert et al., 2002; Mu & Poo, 2006), this suggests that chemoaffinity molecules may not only establish the initial topography of the retinotectal circuit, but can modulate its subsequent refinement as well. Key to the involvement of chemoaffinity cues in retinotopic refinement is their largely unexplored capacity to detect and respond to patterned visual activity, which will be the subject of the next section.

1.1.5 Sensory-dependent mechanisms for retinotopic refinement

While the expression of hardwired gradients appears to be an adequate strategy for the establishment of a crude retinotopic map, so too can visual activity be used as a proxy for evaluating the spatial proximity of neighboring RGC inputs. A common paradigm for studying the role of visual activity in RT development involves the use of the sodium channel blocker tetrodotoxin (TTX) to prevent neuronal firing. Early studies showed that TTX treatment

generated enlarged, and less anatomically precise RGC axon arbors (Meyer, 1983; Schmidt & Buzzard, 1990) preventing RF refinement (Schmidt & Edwards, 1983). These gross morphological defects induced by activity blockade can be partially attributed to alterations in axon arbor dynamics, as TTX treatment increases the relative rate of branch additions to losses (Cohen-Cory, 1999).

Given these morphological defects induced by activity blockade, what is the mechanism that can account for the detection of patterned visual activity? Hebb's proposal that coactive synapses are selectively strengthened prompted a search for molecular candidates that could function as coincidence detectors of neural activity. This search led to the elucidation of the NMDAR, that activates only upon its binding of local glutamate released from the presynaptic cleft, and the simultaneous depolarization of the cell membrane to relieve the Mg^{2+} blockade of its internal pore. Only when these two constraints are satisfied can ions (including Ca^{2+}) flow through the membrane to initiate downstream signaling cascades that recruit AMPARs and other components to stabilize and strengthen the synapse. Like animals treated with TTX, blocking NMDARs results in less precise retinotopic maps (Cline & Constantine-Paton, 1989; Li et al., 2022), and disrupts the functional refinement of RFs (Huang & Pallas, 2001). NMDAR blockade also augments the dynamic additions and subtractions of axon arbors (Rajan et al., 1999), generating more expansive arbors (Schmidt et al, 2000). Experimental manipulations that force RGC afferents from both eyes to innervate the same tectal lobe show that afferents are clustered into eye-specific territories. The separation of afferents from either eye depends on the detection of correlated activity since blockade of NMDARs prevents the ability of RGCs to distinguish same-eye and opposite-eye territories (Ruthazer et al., 2003). Furthermore, the role of NMDARs in Hebbian plasticity was investigated by manipulating activity patterns in mis-projecting

ipsilateral RGCs so that these axons could fire either synchronously or asynchronously with the surrounding contralateral inputs. Synchronous activity patterns were found to stabilize axon branch dynamics, and this effect is abrogated by preventing neurotransmitter release with tetanus toxin (TeNT) or by blocking NMDARs (Munz et al., 2014). Moreover, enhancing NMDAR activation by providing saturating levels of the NMDAR co-agonist D-serine leads to accelerated synaptic functional maturation as well as axonal arbor hyperstabilization (Van Horn et al., 2017).

What occurs downstream of NMDAR activation to promote synapse stabilization? One likely candidate is Ca^{2+}/calmodulin-dependent protein kinase type II (CaMKII). CaMKII can be activated by NMDAR-dependent influx of Ca^{2+} and has been implicated in the regulation of neuronal growth and plasticity (Lisman et al., 2002). Importantly, the expression of a constitutively active CaMKII in postsynaptic tectal neurons reduces arbor complexity in presynaptic axons by reducing the rate of retractions (perhaps owing to more stable synapses) (Zou & Cline, 1996). This same manipulation also contributes to the "unsilencing", of otherwise "silent" synapses that lack AMPARs and constitutes a pivotal process in the maturation of postsynaptic sites (Wu et al., 1996). Conversely, blockade of endogenous CaMKII via the postsynaptic expression of CaMKII-specific inhibitory peptides results in more expansive RGC arbors (Zou & Cline, 1999).

The vital role of NMDARs in detecting patterned activity is further exemplified by their operation in various experience-dependent plasticity mechanisms that contribute to the functional development of the visual circuit. One plasticity mechanism that appears to play an especially prominent role in the developing retinotectal system is spike-timing dependent plasticity (STDP) (Dan & Poo, 2004; Richards et al., 2010). STDP is a form of Hebbian learning that alters

synaptic strength based on the tight temporal correlation of pre- and postsynaptic firing. If a presynaptic neuron fires immediately *prior* to postsynaptic depolarization—and thus contributes to the postsynaptic response—tLTP (timing long-term potentiation) will promote the selective strengthening of synapses, whereas if a presynaptic neuron fires *after* postsynaptic depolarization, it will undergo tLTD (timing long-term depression) to weaken synapses. Indeed, the first *in vivo* evidence for STDP was observed in the retinotectal system of *Xenopus laevis* (Zhang et al., 1998). By precisely controlling the temporal sequence of pre and postsynaptic firing using stimulating electrodes in the retina and OT, Zhang and colleagues demonstrated that synaptic inputs were strengthened if they fired within a 20ms 'potentiation window' before tectal depolarization, and that synaptic inputs were weakened if they fired within a 20ms 'depression window' after tectal depolarization. This group later went on to show that STDP could be induced by visually evoked stimulation, and that NMDARs are essential for the enhancement of compound synaptic currents (CSCs), suggesting that STDP is mediated via postsynaptic NMDARs (Zhang et al., 2000). Since the discovery of STDP in the retinotectal circuit, many studies have demonstrated the involvement of this mechanism in refining functional responses in the tectum. STDP has been shown to contribute to the direction-sensitive shifting of tectal receptive fields (RFs) via a moving bar conditioning stimulus (Engert et al., 2002; Mu & Poo, 2006; Vislay-Meltzer et al., 2006). Importantly, these RF shifts can be abolished by voltage-clamping the postsynaptic neuron—preventing its firing—or blocking NMDARs. This function of STDP has physiological implications for coordinating visually guided behaviours, as it can harmonize retinotopic mapping in accordance with optic flow experienced by forward-swimming tadpoles that shift the visual field from an anterior to posterior direction (Hiramoto & Cline, 2014). This means that tectal responses are not only coordinated across space (i.e., topography),

but across time as well. STDP may also influence the size of tectal RFs, which has implications for visual acuity and the detection of fine spatial gradients. RFs undergo a gradual refinement to become more spatially restricted and more topographically precise (Tao & Poo, 2005). Dong and colleagues (2009) showed that blocking NMDARs with MK-801 prevented the refinement of tectal RFs, resulting in reduced performance for a visual avoidance behavioural assay. Furthermore, STDP appears to be developmentally regulated in albino *Xenopus laevis* tadpoles, suggesting there may be a 'critical period' over which STDP can mediate functional refinement in the developing retinotectal system (Tsui et al., 2010). These studies show that the involvement of NMDARs in STDP-mediated plasticity has important functional consequences for coordinating visually guided behaviours in young tadpoles.

Taken together, these studies demonstrate a role for NMDARs in mediating Hebbian plasticity mechanisms that are essential for the generation of topographically precise maps. However, an unresolved question remains as to how NMDARs, which only detect correlated activity at postsynaptic sites, can relay this information back to the presynaptic arbor to influence its branching dynamics and maturation. The most parsimonious explanation for how postsynaptic NMDARs affects the functional and morphological properties of presynaptic axons is the existence of retrograde cues capable of propagating activity-dependent information back across the synapse. The following section will discuss various molecular candidates for fulfilling this activity-dependent retrograde role.

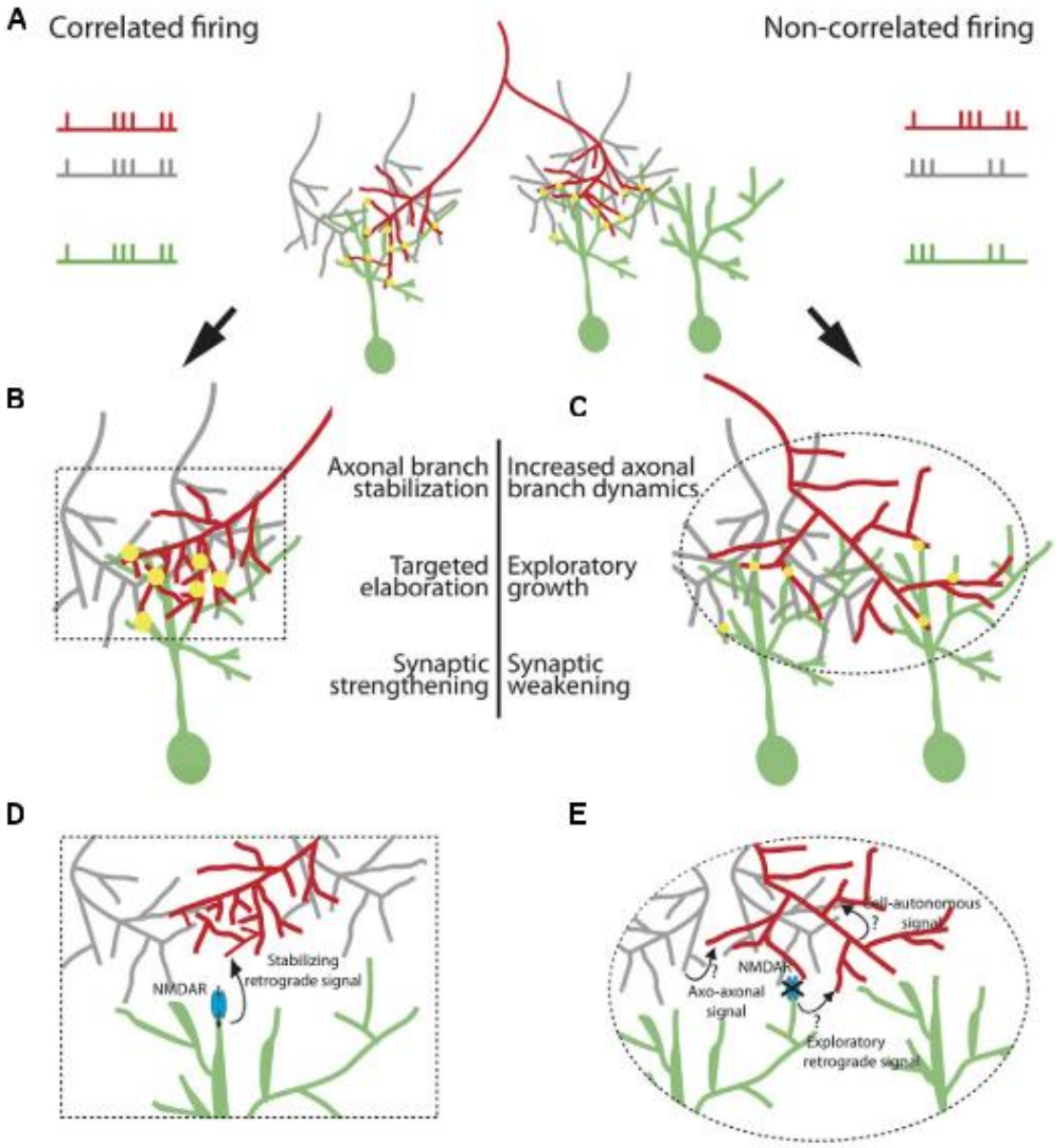

Figure 1.2: Model for how neural activity instructs retinotopic refinement. (A) Under conditions of Hebbian plasticity when an RGC afferent (red) fires in synchrony with its neighbouring axons (grey) to participate in the depolarization of a postsynaptic tectal neuron (green), axon branch dynamics are stabilized, more targeted and have stronger synapses (B). This mechanism likely involves the release of an activity-dependent retrograde cue that stabilizes axon branches downstream of NMDAR activation (D). Conversely, when an RGC fires out of synchrony with its neighbouring inputs, it exhibits increased branch dynamics and exploratory growth while synapses are weakened (C). The increase in axon branch dynamics and synaptic destabilization could be attributed to retrograde signaling, inter-axonal signaling, cell-autonomous signaling, or a combination thereof (E). (Adapted from Kutsarova et al., 2016)

1.1.6 Evidence for activity-dependent retrograde cues regulating axon branch dynamics

The detection of correlated activity patterns by the postsynaptic neuron necessitates a retrograde signal to instruct morphological and physiological remodeling in presynaptic axons (Tao & Poo, 2001). This purported retrograde signal could be either a trans-synaptic adhesion molecule, or a secreted diffusible factor, as long as it satisfies the constraints of being acutely regulated by correlated activity at postsynaptic sites and is transmitted across the synapse to affect presynaptic maturation. Various retrograde candidates have been investigated in the context of retinotopic refinement, however these signals appear to modulate, as opposed to directly mediating activity-dependent remodeling (Schmidt, 2004).

One candidate retrograde messenger that has been well-studied is nitrous oxide (NO). The interest in NO as a retrograde messenger stems from its ability to promote the collapse of RGC growth cones (Renteria & Constantine-Paton, 1996), which could provide a "stop growing" signal required for the stabilization of retinal axons. Furthermore, the enzyme that produces NO, NO synthase (NOS), is Ca^{2+}-dependent and can thus serve as a plausible effector downstream of NMDAR activation. In mammals, NOS is expressed in tectal neurons during retinotopic refinement. Inhibiting NO production in mice was shown to prevent the segregation of ipsilateral/contralateral RGC projections (Campello-Costa et al., 2000; Vercelli et al., 2000). In ferrets, NOS inhibitors prevent the segregation of ON-OFF lamina in the lateral geniculate nucleus (LGN), despite eye-specific segregation not being affected (Cramer & Sur, 1999). NOS inhibitors also fail to prevent the formation or plasticity of ocular dominance columns in the

visual cortex (Finney & Shatz, 1998; Ruthazer et al., 1996). NOS is also present in the OT of frogs during retinotopic refinement (Mize & Lo, 2000; Rentería & Constantine-Paton, 1999). Acute injection of NO donors into the tectal ventricle increases branch additions with no change in subtractions, while NOS inhibitors increase both branch additions and losses (Cogen & Cohen-Cory, 2000). This latter manipulation appears to partially mimic the transient changes in arbor dynamics seen with TTX or NMDAR blockade (Rajan et al., 1999; Schmidt et al., 2000), resulting in longer arbors after 24 hours. However, unlike TTX and NMDAR blockade, treatment with NOS inhibitors failed to prevent the segregation of dually innervated tecta in three-eyed frogs (Rentería & Constantine-Paton, 1999). Taken together these studies suggest that NO may play a more nuanced role in the activity-dependent refinement of visual circuits that may be limited to certain synapses and developmental periods.

Another retrograde candidate that has received considerable attention is brain-derived neurotrophic factor (BDNF). BDNF is a secreted, diffusible factor capable of influencing both axonal and dendritic branching while also being released in an activity-dependent manner (Schinder & Poo, 2000). In the frog visual system, BDNF is expressed in both RGCs and tectal neurons, and its cognate receptor, TrkB, is expressed on RGC axons (Cohen-Cory & Fraser, 1994). Treatment with exogenous BDNF increased the branching complexity of RGC arbors, while inhibiting BDNF with antibodies had an opposite effect of decreasing arbor complexity (Cohen-Cory & Fraser, 1995). Inhibition of BDNF signaling also altered axon dynamics, reducing additions while leaving subtractions unaffected, which is in sharp contrast to the increased additions and subtractions observed with TTX or NMDAR-blockers. This failure of BDNF inhibition to recapitulate the effects of activity blockade is further exemplified by experiments that looked to assess the influence of BDNF on axon arbors in the presence of TTX

(Cohen-Cory, 1999). In the absence of neuronal firing, treatment with anti-BDNF resulted in a normal rate of branch additions with an increase in branch losses, while exogenous BDNF did not significantly alter branch additions or losses. These experiments reveal a complex, nonlinear interaction between BDNF and activity, making it an unlikely candidate for having a direct instructive role in the activity-dependent refinement of RGC arbors.

Together, these studies fall short of convincingly demonstrating a role for these candidates as activity-dependent retrograde signals, leaving open the possibility of other factors playing a more direct role in this process. Despite its involvement in early retinotopic mapping, Wnt signaling is a promising, albeit understudied, candidate for mediating activity-dependent circuit refinement. The following section will review the mechanisms and functions of Wnts that make them plausible candidates as activity-dependent retrograde factors.

1.2 Wnt Signaling

1.2.1 Introduction to Wnt signaling

It should be appreciated that biological organisms, in all their complex and variegated forms, share a common origin in single-celled organisms. The events precipitating the progression from single to multicellular life are far from trivial, requiring the careful coordination of cellular units into complex arrangements that are more than the sum of their parts. For such a transition to take place requires the evolution of intercellular communication systems that enables cells to transmit and receive information from one another. Deciphering these elaborate communication systems—expressed in a language of cell-surface and secreted molecules—holds the key to understanding the evolutionary history and development of multicellular organisms.

One family of factors that likely had a preeminent role in the development of multicellular life are Wnts. The name "Wnt" is derived from the coincidental discovery of the Wingless gene in Drosophila and the int-1 gene in mice that were later identified as homologs sharing a common evolutionary origin (Rijsewijk et al., 1987). The family of Wnt genes has since expanded to include 19 distinct genes that are separated into 12 conserved categories, in mammals (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/). These genes are remarkably well conserved across multicellular organisms, but are notably absent from single-celled organisms, suggesting they played a significant role in the evolution of multicellular life (Kusserow et al., 2005; Petersen & Reddien, 2009). Wnt genes are classified according to a conserved cysteine-rich sequence that gets post-translationally modified to include a palmitoyl

group. The addition of this lipophilic palmitoyl group confers what is thought to be a universal property of Wnts—their hydrophobicity—that renders them uniquely suitable as short-range cell-to-cell signaling factors (Willert et al., 2003). The palmitoylation of Wnts by the O-acyltransferase Porcupine appears to be essential for the secretion and signaling properties that distinguish Wnts (Kadowaki et al., 1996; Takada et al., 2006). The mechanisms governing the secretion of Wnt proteins are still largely unresolved, however substantial progress has been made in identifying key regulators of this process. Due to their hydrophobicity, Wnts have difficulty diffusing in aqueous environments over long ranges, so the shielding of their palmitoleate moiety is required (Langton et al., 2016). One key player in the secretion of Wnts is the type II multipass transmembrane protein called Evenness Interrupted (Evi). Evi binds to lipid-modified Wnts to aid in their trafficking to the cell-membrane, as well as their secretion into the extracellular environment by inserting into small extracellular vesicles called exosomes that function as carriers for Wnt proteins (Gross et al., 2012; Koles et al., 2012; Korkut et al., 2009). Wnts may also associate with lipoparticles or lipoproteins such as Swim (Secreted Wnt-interacting molecule) (Mulligan et al., 2012), high-density lipoprotein (Neumann et al., 2009), and Afamin (Mihara et al., 2016).

Despite Wnts having lipid modification in common, they can have widely divergent and multifarious functions in orchestrating cellular processes including proliferation, differentiation, migration, and apoptosis. Frizzleds (Fz/Fzd) are seven-pass transmembrane proteins that function as the cognate receptors for Wnt proteins, and act as the primary transducers of Wnt signaling cascades. The Fz family of receptors share a common cysteine-rich domain (CRD) that binds to extracellular Wnts. There are 10 Fz homologs in mammals that appear to bind to different Wnts at different affinities and can preferentially signal through distinct Wnt-dependent biochemical

cascades (Wang et al., 2016). The promiscuity of Wnt/Fz interactions and the often redundant expression of different Wnt and Fz homologs makes it especially difficult to discern the distinct functional effects of Wnt and Fz genes. There are also various Wnt co-receptors like lipoprotein receptor-like proteins 5 and 6 (LRP5/6), Ryk and tyrosine kinase-like orphan receptors (RoRs) that transduce distinct downstream Wnt signaling events. The diversity of Wnts and their receptors—not to mention their vast array of downstream effectors—endows these signaling molecules with an unparalleled versatility in facilitating intercellular communication.

Adding to the complexity of Wnt signaling is the fact that Wnts and their pathway-specific components are highly contextual, as they are dynamically regulated across space and time. This means that the same Wnt may not have the same function in two different cells at the same time, nor will it have the same function in the same cell at two different times. Thus, Wnt signaling may be more accurately conceived as a vast network of dynamically interacting and overlapping components, as opposed to tightly regulated mechanistic pathways (Kestler & Ku, 2008). Despite the inherent difficulty in delineating the multifaceted functions of such a complex pathway, there is still much to gain from understanding the roles of Wnt signaling in regulating developmental processes. The following section will look to unpack the detailed mechanisms of the main Wnt signaling pathways.

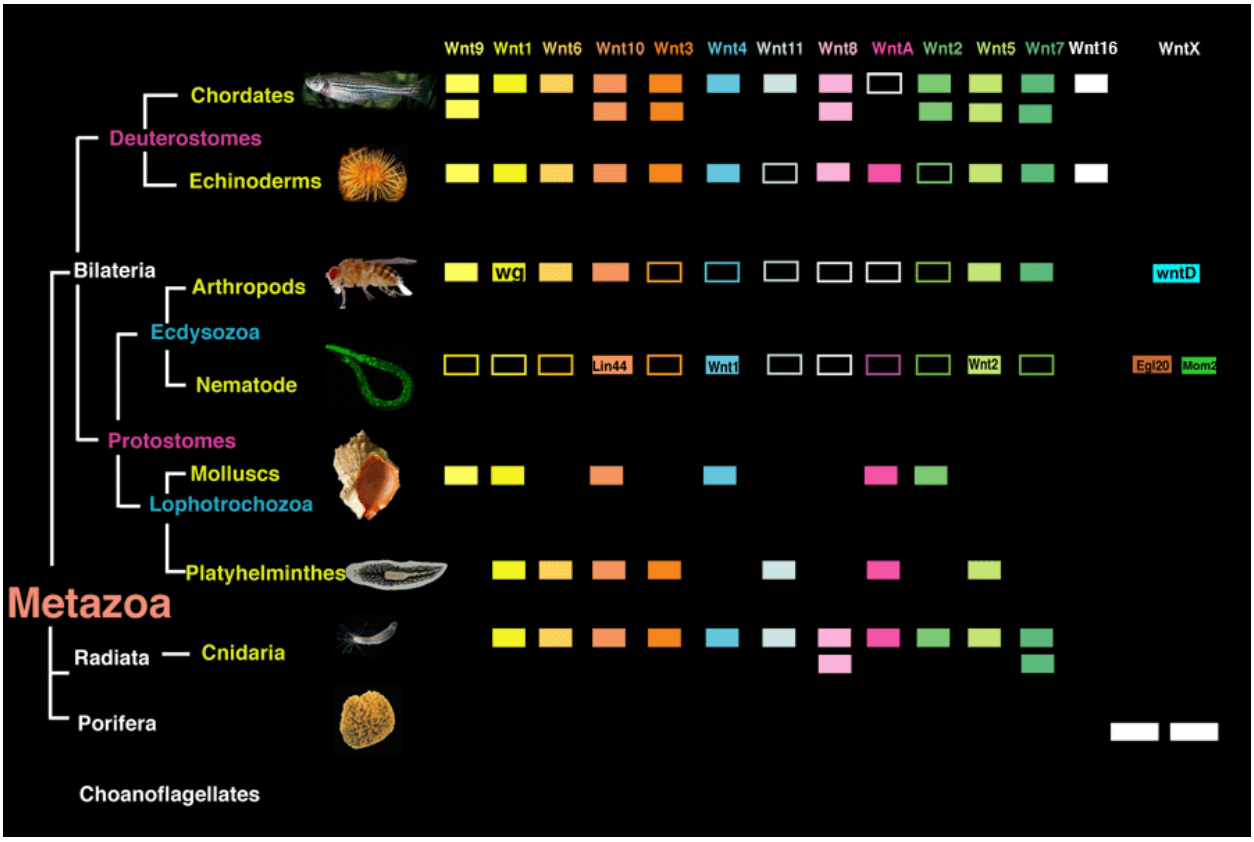

Figure 1.3: Evolutionary conservation of Wnts across Metazoan lineages. (From the Nusse Lab Wnt Homepage http://web.stanford.edu/group/nusselab/cgi-bin/wnt/)

1.2.2 Wnt signaling pathways

Wnt signaling occurs through three primary pathways: the canonical/ β-catenin pathway, the planar-cell polarity (PCP) pathway, and the calcium pathway (**Fig. 1.4**). The most well characterized of these pathways is the "canonical" β-catenin pathway that is initiated upon the binding of extracellular Wnts to Fzs, promoting an association with Fz and its co-receptor, LRP5/6. This interaction translocates the versatile scaffolding protein Dishevelled (Dsh/Dvl) to the plasma membrane via an interaction with its PDZ domain and the cytoplasmic domain of Fz (Wong et al., 2003). Dvl transduces downstream signaling by recruiting axin through their mutual DIX domains (Kishida et al., 1999). Axin is an integral component of the β-catenin

destruction complex, forming in association with glycogen synthase kinase 3β (GSK3β), adenomatous polyposis coli (APC) and casein kinase 1ε (CK1ε), which phosphorylates β-catenin to target it for proteolysis. Therefore, the disruption of the β-catenin destruction complex caused by the binding between Dvl and Axin results in the accumulation of β-catenin, allowing it to translocate to the nucleus where it regulates transcription of Wnt target genes by activating the T- cell factor/ lymphoid-enhancer factor (TCF/LEF) family of transcription factors (Molenaar et al., 1996). β-Catenin activates TCF by replacing the transcriptional repressor, Groucho, that normally blocks transcription of Wnt target genes (Daniels & Weis, 2005). Furthermore, β-catenin interacts with histone acetylases such as CREB-binding protein (CBP)/p300 and the SWI/SNF complex member Brg-1 which facilitates the remodeling of chromatin proximal to TCF binding sites (Hecht et al., 2000; Takemaru & Moon, 2000). The activation of this transcriptional pathway targets many genes, evident from the diverse range of phenotypes—often involving developmental patterning—that result from canonical pathway disruption (Cadigan & Nusse, 1997; Clevers & Nusse, 2012). One key element of this pathway is that it regulates the transcription of its own components, enabling pathway-specific feedback control (Logan & Nusse, 2004). Canonical pathway activation has been shown to promote axin expression (Jho et al., 2002), which is a negative regulator of canonical signaling, thus forming an auto-inhibitory feedback loop.

Aside from regulating the transcription of its own components, the transcriptional targets of canonical Wnt signal are both highly diverse and context-specific (Logan & Nusse, 2004). It is estimated that as little as 5% of transcriptional targets are shared between different cell-lineages (Vlad et al., 2008). This is considering that Wnts can induce or repress the transcription of hundreds to thousands of genes (a list of target genes can be found here:

https://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes). Adding to this complexity, the transcription of Wnt target genes can have higher-order effects beyond their direct targets by regulating transcriptional factors, as well as regulators of separate signaling pathways. Despite this complexity, there appear to be highly represented functional classes of genes that are regulated by Wnt signaling including genes related to proliferation, apoptosis, and cell-cycle regulation, among others (Vlad et al., 2008). Thus, the diversity and context-specificity of Wnt transcriptional targets reflect the versatile cellular functions controlled by Wnt signaling.

In addition to regulating transcription, effectors of the Wnt/β-catenin pathway can initiate divergent signaling cascades that play prominent roles in regulating synaptic processes including axon guidance, neurotransmitter release, and synaptogenesis (Mulligan & Cheyette, 2016; Salinas, 2012). Many of the familiar players that comprise the β-catenin destruction complex, including β-catenin itself, have auxiliary transcription-independent functions. These include interactions with cytoskeletal components, cellular adhesion molecules, and synaptic vesicles. The functional implications of these various divergent mechanisms will be discussed in a later section.

In addition to the canonical Wnt signaling pathway, there are two non-canonical, β-catenin-independent pathways: the Wnt/PCP pathway and Wnt/Ca^{2+} pathway. The PCP (planar cell polarity) pathway (alternatively called the Wnt/JNK pathway) is aptly named for its involvement in establishing the polar orientation of epithelial tissues that was first observed in Drosophila (Seifert & Mlodzik, 2007). Like the canonical pathway, the core components for transducing Wnt/PCP signaling are Fz and Dsh. However, signaling through the Wnt/PCP pathway also requires the receptor tyrosine kinase ROR2, which binds to extracellular Wnts to initiate a distinct biochemical cascade (Oishi et al., 2003). It does so by recruiting Dsh, which

serves as the conductor between these various Wnt pathways, that then binds to and activates the adaptor protein Daam1 (Liu et al., 2008). Together, Dsh and Daam1 recruit the Rho guanine exchange factor WGEF (weak-similarity GEF) forming the basis of the Rho-GTP complex which subsequently activates ROCK kinase to remodel the cytoskeleton (Habas et al., 2001). Daam1 further contributes to cytoskeletal remodeling through an interaction with the actin-binding protein Profilin1 (Sato et al., 2006(a)). Dsh can also mediate a parallel signaling cascade in this pathway through an association with another small Rho GTPase, Rac, resulting in the activation of c-Jun N-terminal kinase (JNK). Activated JNK can either remodel the actin cytoskeleton directly (Rosso et al., 2005) or translocate to the nucleus to regulate the transcription of target genes in this pathway (Schambony & Wedlich, 2007). An interesting consequence of signaling through the Wnt/PCP pathway, is its ability to exert an antagonistic effect on the Wnt/β-catenin pathway (Komiya & Habas, 2008a). One study showed that Wnt5A signaling could be diverted through the β-catenin or PCP pathway by overexpressing Fz4 and LRP5 or ROR2, respectively (Mikels & Nusse, 2006). This suggests that the pathway-specific activation of any given Wnt ligand is highly sensitive to the complement of receptors expressed, allowing for a tight control of these pathways based on the cellular context.

The other non-canonical Wnt pathway, the Wnt/Calcium pathway, was discovered when RNA encoding Wnt and Fz proteins was injected into zebrafish and Xenopus embryos and found to induce intracellular calcium release (Slusarski et al, 1997(a); Slusarski et al., 1997(b)). Signaling through this pathway is also transduced through ROR2 (Hikasa et al., 2002; McQuate et al., 2017) and Fzs which recruit Dsh via its PDZ and DEP domains, whereas the DIX domain is dispensable for intracellular Ca^{2+} influx (Tada & Smith, 2000). This interaction involves the recruitment of heterotrimeric GTP-binding proteins via Fzs which act through Dsh to then

activate phospholipase C (PLC) and phosphodiesterases (PDEs), catalyzing an IP3/DAG-dependent release of Ca^{2+} from internal stores in the endoplasmic reticulum (ER) (Slusarski et al., 1997(a)). The downstream effects induced by the release of Ca^{2+} are multifarious, causing the activation of various Ca^{2+}-dependent enzymes including CaMKII, protein kinase C (PKC), and calcineurin (Komiya & Habas, 2008; Mulligan & Cheyette, 2016). Activation of CaMKII results in signaling through a TAK1-NLK pathway that, like the Wnt/PCP pathway, antagonizes β-catenin/TCF transcription through the canonical pathway (Ishitani et al., 1999). The activation of PKC phosphorylates the small GTPase Cdc42 to remodel the actin cytoskeleton (Winklbauer et al., 2001). Furthermore, calcineurin promotes transcriptional regulation through the transcription factor nuclear factor of activated T-cells (NFAT), which also has a role in antagonizing canonical Wnt signaling (Saneyoshi et al., 2002).

Although this covers the three main Wnt signaling pathways, it should be noted that there are various other pathways that play a more specified functional role. One especially pertinent pathway is mediated through the atypical receptor tyrosine kinase Ryk, which plays a prominent role in axon guidance and neurite outgrowth (Lu et al., 2004; Yoshikawa et al., 2003). Ryk is conserved across species and generally mediates repulsive axon guidance, but the signaling cascades by which it does so appear to differ based on the circuit and species. For example, mammalian Ryk signaling involves some degree of overlap between Wnt/PCP and Wnt/Ca^{2+} pathways whereas the Ryk homolog in Drosophila, Derailed, likely signals via a completely independent pathway that doesn't require essential Wnt components like Fz and Dvl (Fradkin et al., 2009). A more detailed description of the functions and mechanisms of Ryk signaling will be provided in a later section.

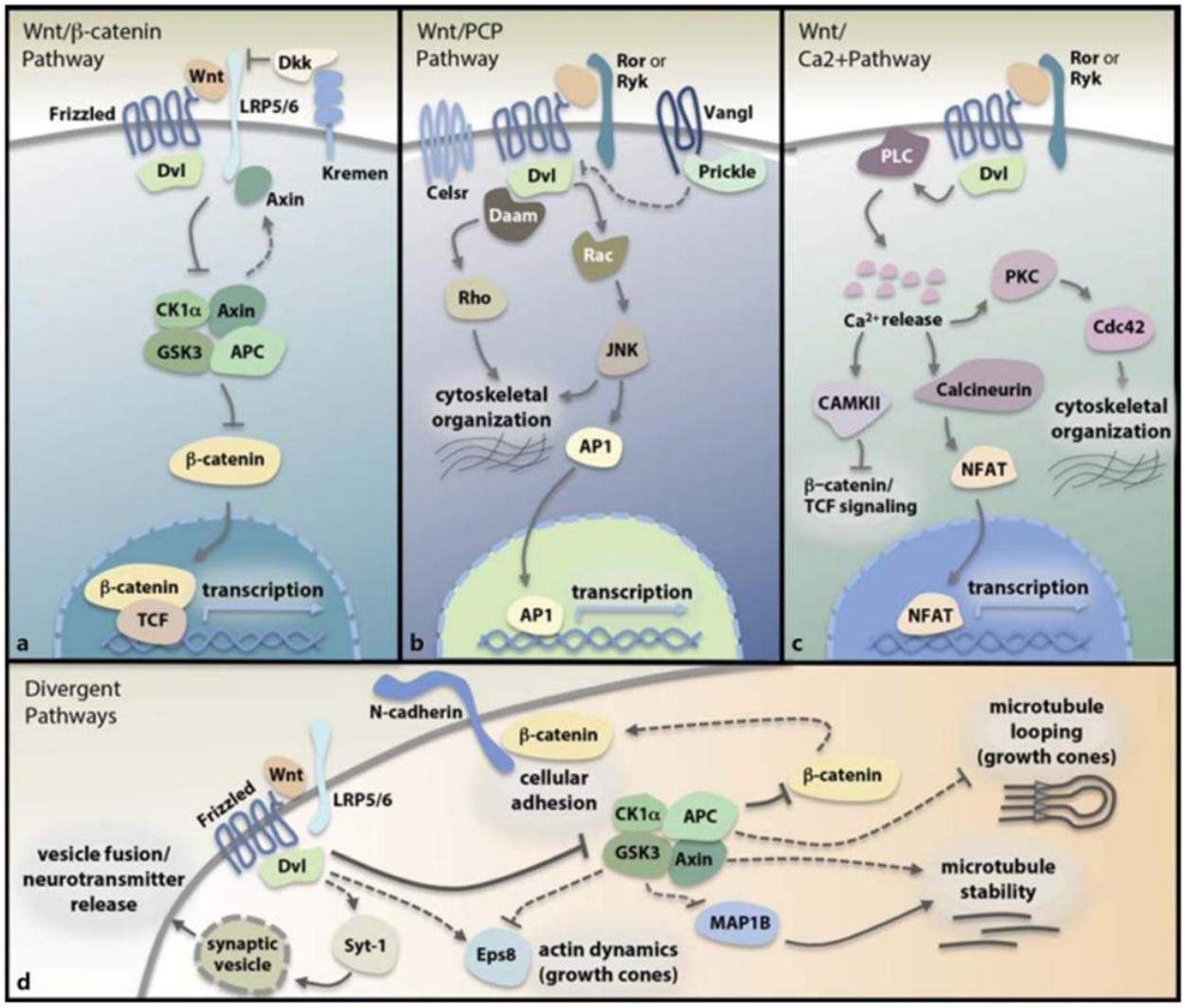

Figure 1.4: Summary of Wnt signalling pathways (A) The canonical Wnt/β-catenin pathway. (B-C) Non-canonical Wnt pathways. (D) Transcription-independent divergent canonical Wnt pathways. (Figure adapted from Mulligan & Cheyette, 2016)

1.2.3 Wnt functional roles

Given the ubiquity of Wnts and their cell-type/tissue specific expression, it will come as no surprise that Wnt signaling has a vast array of functional consequences on organism development and homeostasis. One way to assess the functional importance of Wnt signaling is its connection to developmental disorders and disease. In humans, there are various diseases that

are linked to mutations in Wnt signaling components (Clevers & Nusse, 2012; MacDonald et al., 2009). Mutations in Wnt signaling components such as axin, APC, β-catenin, TCF4 and LEF1 are especially prevalent in the development of cancers, owing to the role of Wnt signaling in cell differentiation, proliferation, and cell migration. Perhaps more relevant, the dysregulation of Wnt signaling genes has also been implicated in various neurodevelopmental disorders, including schizophrenia, autism spectrum disorder, and bipolar disorder (Mulligan & Cheyette, 2016), and neurodegenerative diseases including Alzheimer's and Parkinson's Disease (Inestrosa & Arenas, 2010; Inestrosa & Varela-nallar, 2014; Noelanders & Vleminckx, 2016). As these neurological disorders stem from aberrations in neural homeostasis, neurogenesis, and synapse connectivity, their association with dysfunctional Wnt components highlights this signaling system as vital for supporting healthy brain development and maintenance.

In addition to observational studies, early mutagenesis experiments done in Drosophila revealed a critical role for Wnt signaling components in developmental patterning (and hence the names for these cognate components, like "Wingless", "Frizzled", "Porcupine", "Armadillo" and "Disheveled", reflective of their respective phenotypes) (Perrimon & Mahowald, 1987). Another model that has figured prominently in the study of Wnt signaling in early morphogenesis is *Xenopus laevis*, which due to their large, externally developing eggs, is conducive to RNA injections of Wnt components which resulted in striking developmental phenotypes. These phenotypes appeared to be caused by a disruption of specific Wnt pathways, with the canonical pathway leading to the formation of a secondary body axis (splitting the neural tube to generate a dorsal and ventral head), and the Wnt/PCP pathway disrupting convergent extension movements during gastrulation in Xenopus embryos (Rothbacher et al., 2000; Sokol, 1996; Wallingford et al., 2000). These studies were instrumental for elucidating the mechanisms responsible for

pathway specific regulation of Wnt signaling, with the universal Wnt signaling factor Dsh playing a pivotal role in differentially mediating these pathways. With regards to nervous system development, the functions of Wnt signaling in neural morphogenesis are widely conserved in vertebrates, contributing to neural tube formation, neural plate specification, neurogenesis, and neural precursor migration (Mulligan & Cheyette, 2016). At later stages of brain development, Wnts and their effectors are expressed in a region-specific and time-dependent manner, suggesting their functional roles are varied and constrained within neural circuits. Not only is Wnt signaling essential for global neural patterning, but it has well-characterized roles in synapse formation and function, which will be the topic of the next section.

1.2.4 Wnts at the synapse

There is an abundance of evidence demonstrating that Wnts are essential intercellular signaling factors for directing synapse formation and maintenance (Ciani & Salinas, 2005; Inestrosa & Arenas, 2010; Salinas, 2012). The first evidence for the synaptogenic effects of Wnts was observed in the mouse cerebellum, where Lucas and Salinas (1997) demonstrated that Wnt-7a, expressed in postsynaptic granule cells, regulates axon branching and the clustering of the presynaptic marker synapsin-1 in presynaptic mossy fibers. They went on to show that inhibiting Wnt signaling with the Wnt antagonist sFRP-1 or generating a Wnt-7a mutant mouse delayed the morphological maturation and synapse formation of mossy fiber terminals (Hall et al., 2000), providing the first concrete evidence that Wnt signaling is essential for synaptogenesis and functions as a target-derived retrograde factor to instruct axonal growth cones. This retrograde action of Wnts has since been discovered to play a role in axon guidance and branching across a diverse range of species and neural circuits. Wnt-3 acts in a retrograde manner to induce axon

pausing and terminal arborization in a subset of dorsal root ganglion (DRG) sensory neurons (Krylova et al., 2002). In Drosophila, Wnt5 repels commissural axons in the DRG via the Ryk ortholog Derailed (Yoshikawa et al., 2003), whereas in mice, Wnt-4 acts as an attractive guidance cue for commissural axons in a Fz3-dependent manner (Lyuksyutova et al., 2003; Wang et al., 2002). The involvement of Fz3 in axon guidance extends to various other axon tracts in the mouse CNS (Hua et al., 2014). More generally, Wnt signaling contributes to axon guidance in corticospinal tracts (Liu et al., 2005), dopaminergic circuits (Fenstermaker et al., 2010) the striatum (Morello et al., 2015), the corpus callosum (Hutchins et al., 2010; Keeble et al., 2006), and RGCs (Schmitt et al., 2006), operating through various pathways and acting as both attractive and repulsive cues.

What are the molecular mechanisms that account for the influence of Wnts on axon growth cones? Wnt signaling can regulate microtubule (MT) stability and dynamics through Dsh, which inhibits GSK3β causing a decrease in MAP-1B (microtubule associated protein-1B) phosphorylation that stabilizes MTs (Ciani et al., 2004; Krylova et al., 2000). This effect is mediated through a divergent canonical pathway that functions independently of β-catenin transcriptional regulation. This divergent pathway also appears to be conserved in Drosophila. Wnt signaling through the canonical co-receptor LRP5/6 and the Drosophila homolog of GSK3, *shaggy*, promotes the formation of MT "loops" within presynaptic boutons that influence axon branching and presynaptic assembly at the neuromuscular junction (NMJ) (Franco et al., 2004; Miech et al., 2008). Another component of the β-catenin destruction complex, APC, interacts with the plus ends of MTs to promote the directional extension of axon growth cones (Purro et al., 2008). The activation of Wnt signaling causes the translocation of APC to Dvl docking sites at the plasma membrane, resulting in a loss of MT directionality in growth cones. This

effectively halts the forward advance of the growth cone, causing it to increase in size and extend axonal branches—a precondition for synapse formation. Wnt signaling through the Ryk receptor also plays an important role in repelling axon growth cones. In rodents, Wnt5a signaling through the Ryk receptor promotes both the outgrowth and repulsive guidance of cortical axons (Keeble et al., 2006; Li et al., 2009). In this system, Ryk activation signals through the Wnt/Ca^{2+} pathway to promote Ca^{2+} influx through transient receptor potential (TRP) channels in an IP3-dependent manner (Hutchins et al., 2010). This causes the downstream activation of CaMKII which plays an essential role in axon outgrowth and guidance by inhibiting the MT stabilizing protein, tau (Li et al., 2014). In Drosophila, Wnt5/Ryk signaling appears to promote axon repulsion through a separate mechanism involving the recruitment of active Src family kinases (SFKs) (Wouda et al., 2008). Although this pathway appears to be distinct from Ryk signaling in mammals, the binding of c-Src to mammalian Ryk suggests this pathway could be conserved in mammals as well (Fradkin et al., 2009).

In addition to MTs, Wnt signaling regulates another key component of the cytoskeleton, F-actin, which plays a prominent role in the dynamic remodeling of synaptic sites (Chia, Patel, & Shen, 2012; Van Aelst & Cline, 2004). Wnt3a increases F-actin accumulation and dynamics in DRG growth cones via another divergent pathway involving Dvl1 and GSK3β. An interaction between the PDZ domain of Dvl1 with the actin binding protein Eps8 (epidermal growth factor receptor pathway substrate 8) is responsible for the Wnt3a-mediated increase in F-actin dynamics (Stamatakou et al., 2015). In *C.Elegans,* the Wnt homologs Lin-44 and EGL-20 locally inhibit presynaptic formation of PLM mechanosensory neurons to generate a highly typified arrangement of synapses along the anterior-posterior commissure (Klassen & Shen, 2007; Pan et al., 2006). Wnts regulate synaptic specification by spatially restricting F-actin accumulation to

future sites of presynaptic branching and formation, via the Wnt/PCP component, Vang-1, which locally inhibits Rho and Rac GTPases (Chen et al., 2017).

The influence of Wnts on neural circuit formation goes beyond just axon guidance, as they can also directly contribute to presynaptic assembly and maturation. Mice with a double knockout (KO) for Dvl1/Wnt7a display reduced localization of presynaptic markers at mossy fiber terminals which reduces mEPSC frequency at these synapses, indicating a defect in neurotransmitter release (Ahmad-Annuar et al., 2006). Presynaptic terminals in the hippocampus of Dvl1/Wnt7a KO mice also show reduced synaptic transmission owing to a smaller pool of readily releasable synaptic vesicles and reduced SNARE complex formation (Ciani et al., 2015). This effect of Wnt signaling on synaptic vesicle recruitment and transmission is mediated by a direct interaction between Dvl1 and synaptotagmin-1. Moreover, treatment of hippocampal slices with exogenous Wnt7a increases synaptic vesicle clustering and release as shown by an increase in FM dye uptake and mEPSC frequency while decreasing PPR (Cerpa et al., 2008). Wnt7a also promotes the clustering of presynaptic nicotinic acetylcholine receptors (nAChRs) in the hippocampus by modulating the localisation of APC (Farías et al., 2007). Enhancing canonical Wnt signaling by applying Wnt agonists (Beaumont et al., 2007) or exogenous Wnt3a (Avila et al., 2010) results in an acute increase in excitatory neurotransmission. Wnt3a has been shown to induce Fz1-mediated synaptogenesis in cultured hippocampal neurons (Varela-Nallar et al., 2009). Interestingly, the effects of Wnt signaling on presynaptic function appear to be pathway specific, as Wnt ligands that preferentially activate the canonical pathway (Wnt7a, Wnt7b and Wnt3a) enhance synaptogenesis whereas the noncanonical Wnt ligand, Wnt5a, inhibits synaptogenesis in the hippocampus (Davis et al., 2008). Even in the absence of Wnt signaling, Wnt effectors such as β-catenin have been shown to interact with the trans-synaptic adhesion

molecule N-cadherin, which contributes to the recruitment of synaptic vesicles (Bamji, 2005; Bamji et al., 2003).

Wnt signaling also has well-characterized functions at the other side of the synapse, regulating dendritogenesis and postsynaptic differentiation. An early study using cultured mouse hippocampal neurons showed that Wnt7b promotes dendritogenesis by activating the Wnt/PCP components Rac and JNK (Rosso et al., 2005). More recently, it was shown that this effect of Wnt7b on dendritic arborisation occurs through a non-canonical Fz7 pathway that also activates CAMKII—an essential component of the Wnt/Ca^{2+} pathway—suggesting some degree of overlap between PCP and Ca^{2+} non-canonical pathways (McLeod et al., 2018). The more classic non-canonical Wnt ligand, Wnt5a, also has a considerable role in spine morphogenesis and neurotransmission. Wnt5a was first shown to act through PCP components to promote the clustering of postsynaptic density protein-95 (PSD-95) at postsynaptic junctions in hippocampal neurons (Farías et al., 2009). Postsynaptic Wnt5a signaling also induces Ca^{2+} influx to enhance spontaneous and excitatory neurotransmission via the recruitment of NMDARs (Varela-Nallar et al., 2010). This Wnt5a-mediated potentiation of NMDAR currents not only affects basal synaptic transmission but long-term potentiation (LTP) as well (Cerpa et al., 2011). Both the Wnt/ Ca^{2+} effector, PKC, and the Wnt/PCP effector, JNK, appear to be involved in this signaling cascade, providing another example of crosstalk between non-canonical Wnt pathways. Another important component of non-canonical Wnt5a signaling in dendrites is the atypical receptor RoR2. The subcellular localization of RoR1/2 is developmentally regulated and crucial for the formation of hippocampal synapses (Paganoni et al., 2010; Paganoni & Ferreira, 2003). The binding of Wnt5a to RoR2 activates PLC and voltage-gated calcium channels (VGCCs) to increase intracellular Ca^{2+} levels, which in turn activate PKC and JNK to promote the SNARE-

dependent trafficking of NMDARs to active synapses (Cerpa et al., 2015; McQuate et al., 2017). Whereas Wnt/ Ca^{2+} signaling modulates receptor trafficking and synaptic transmission, the Wnt/PCP pathway plays a more prominent role in postsynaptic stabilization and maturation by recruiting N-cadherin to the postsynaptic density (PSD) via Vangl2 (Nagaoka et al., 2014; Okerlund et al., 2016). Furthermore, Wnt3 promotes the rapid clustering of AChRs in the mouse NMJ via Rac1 signaling (Henriquez et al., 2008). However, in the NMJ of Drosophila Wnts promote postsynaptic differentiation by an altogether different mechanism, which involves the Wnt-induced cleavage of the C-terminal tail of DFz2, which then gets imported into the nucleus to regulate transcription (Ataman et al., 2006; Packard et al., 2002). On the other hand, Wnt signaling through the Ryk receptor acts as a negative regulator—as opposed to a positive regulator—of dendritogenesis in mouse hippocampal and cortical neurons (Lanoue et al., 2017). Finally, the effects of Wnt signaling on postsynaptic differentiation and function do not appear to be exclusive to excitatory synapses, as Wnt5a regulates GABA-A receptor recycling in hippocampal neurons (Cuitino et al., 2010).

Together, these studies give a glimpse into the varied and diverse roles that Wnts play in regulating synaptic connectivity at both sides of the synapse. The next section will explore the contribution of Wnt signaling in the regulation of activity-dependent synaptic plasticity.

1.2.5 Wnts regulate synaptic plasticity

In addition to their synaptogenic functions, Wnt signaling is both a regulator of, and regulated by activity-dependent mechanisms at the synapse. The depolarization of cultured hippocampal neurons was shown to stimulate the release of Wnts, which act through a β-

catenin/N-cadherin complex to enhance dendrite arborization (Yu & Malenka, 2003). Another study showed that neuronal activation via NMDARs promotes the transcription of CREB-responsive genes, one of which is Wnt2 that gets synthesized and released in an activity-dependent manner to promote dendrite arborization (Wayman et al., 2006). This increase in Wnt2 transcription could be mediated by activity-dependent BDNF release that was shown to upregulate Wnt2 expression (Hiester et al., 2013). Not only can BDNF promote Wnt gene expression, but Wnts have also been reported to regulate BDNF expression in RGCs (Yi et al., 2012), revealing an interdependency and overlap of these different plasticity factors.

Tetanic stimulation of hippocampal neurons has also been shown to promote an NMDAR-dependent release of Wnt3a which facilitates LTP (Chen et al., 2006). A more recent study showed that LTP-mediated dendritic spine plasticity requires Wnt-Fz7 signaling (McLeod et al., 2018). They show that inducing LTP elevates Wnt7a/b proteins at the synapse, resulting in the activation of CaMKII, PKA and ERK (extracellular-signal-regulated kinase) causing the acute localization of AMPARs to the synapse. Evoked activity also stimulates the release of Wnt1 from presynaptic boutons in the Drosophila NMJ, initiating both pre and postsynaptic signaling cascades via distinct mechanisms (Ataman et al., 2008). Furthermore, neuronal activity promotes the localization of Fz5 receptors to both pre- and postsynaptic sites, mediated by Wnt7a. High frequency stimulation (HFS) increases the mobilization of Fz5 to the cell membrane at synapses, whereas low frequency stimulation (LFS) decreases Fz5 trafficking and localization at synapses (Sahores et al., 2010). The increase in synaptogenesis caused by HFS is inhibited by the Wnt scavenger Fz5CRD, showing Wnt signaling acts as a permissive, activity-dependent signal for synaptogenesis.

Wnt signaling may also contribute to synaptic plasticity in a more indirect manner by promoting the synthesis and release of other plasticity factors like NO. In the hippocampus, NOS associates with PSD95 enabling the retrograde action of NO that activates soluble guanylyl cyclase in presynaptic boutons (Nikonenko et al., 2008). Wnt5a was shown to directly regulate NO production through the Ca^{2+} pathway which leads to the insertion of GluN2B subunits (Muñoz et al., 2014). Wnt7a also increases NOS activity to modulate excitatory synaptic transmission in a Dvl-dependent manner (McLeod et al., 2020).

As one would expect, the influence of Wnts in synaptic plasticity also has important implications for behavioral learning. Subjecting mice to an enriched environment (EE) and enhancing excitatory activity increased Wnt7a/b levels in the hippocampus which promoted an increase in hippocampal synapse number (Gogolla et al., 2009). This EE-mediated increase in synapse number could be prevented by inhibiting Wnt signaling with sFRP-1, while Wnt7 treatment mimicked EE *in vivo*, showing Wnt signaling is a necessary and sufficient factor for enhancing synaptogenesis in adult rats. Other studies confirmed that Wnt7 increases in the hippocampus after spatial learning (Tabatadze et al., 2012), and that trafficking of Wnt7 to dendritic spines is an activity-dependent process, which retrogradely influences presynaptic differentiation by increasing bassoon-marked active zones (Tabatadze et al., 2014). Canonical Wnt signaling is required for hippocampal memory consolidation (Fortress et al., 2013) as well as long-term fear memory consolidation in the amygdala (Maguschak & Ressler, 2011). The deletion of Wnt5a causes deficits in dendritogenesis in the hippocampus, resulting in impaired spatial learning in adult mice (Chen et al., 2016).

These studies provide incontrovertible evidence that Wnts modulate synaptic plasticity, but if and how these functions are operant during retinotectal development remain unexplored.

1.2.6 Wnts in the retinotectal system

The Wnt literature reviewed so far derives mostly from studies done in other animal models and neural circuits, but what is the role of Wnt signaling in establishing synaptic connectivity in the retinotectal circuit? The functions of Wnts in retinotectal development have been touched on in previous sections, but here I will synthesize these findings to give a general idea for how Wnts may instruct retinotopic mapping.

In *Xenopus laevis* tadpoles, Wnt signaling components are developmentally regulated and exhibit region-specific expression throughout the body and in the brain (Session et al., 2016). An early in-situ hybridization study revealed localized XWnt3A expression—and to a lesser extent XWnt1—in the midbrain region of stage 31 embryos (Wolda et al., 1993). At this early developmental period, Wnt3A is expressed in a D-V decreasing gradient, which is consistent with a study showing graded Wnt3 expression in the OT and SC of chicks and mice, respectively (Schmitt et al., 2006). A more recent study showed that this graded pattern of XWnt3A expression in the OT persists in stage 45 tadpoles—a key developmental time period for the remodeling of retinotectal synapses (Lim et al., 2010). Given the influence of Wnt3/3A signaling in establishing topographic mapping in chicks and mice, we presume that Wnt3A signaling plays a similar function in guiding RGC axons along the D-V axis of the *Xenopus laevis* OT via Ryk and Fz receptors in RGC growth cones. However, beyond the role of Wnt3A in mediating axon guidance, Lim et al. (2010) reported that Wnt signaling, alongside EphB/ephrin-B signaling, contributes to region-specific RF plasticity induced by an experience-dependent conditioning stimulus. They showed that perfusing the Wnt antagonist sFRP2 inhibited RF shifts in the dorsal region of the tectum, where Wnt3A expression is highest. They also showed that overexpressing Wnt3A postsynaptically, but not presynaptically, enhances RF shifts in the ventral tectum where

Wnt expression is low. Postsynaptic Wnt3A expression also enhanced the conditioning stimulus-induced changes in the spiking ensemble of presynaptic RGC inputs, which were more pronounced in the ventral tectum. Together, these results provide tantalizing clues for the involvement of retrograde Wnt signaling in the activity-dependent refinement of tectal RFs.

However, it remains to be understood whether Wnt signaling has a direct role in regulating neural morphology and/or synaptic function during retinotectal modeling, as well as the mechanisms through which it acts. This research seeks to clarify the role of Wnt signaling during retinotectal modeling and has important implications for how signaling pathways can be dynamically regulated to coordinate circuit development.

1.3 Approaches and Limitations for Investigating Synaptic Function

With the goal of this project being to elucidate the function and mechanisms of Wnt signaling during synaptic development, it will be useful to first examine the methods and techniques used to ascertain changes in synaptic strength. Synapses exhibit a tremendous capacity to change the strength and number of their connections, which forms the basis of learning and memory in the brain. Our ability to investigate and model synaptic plasticity is due, in large part, to advances in electrophysiological techniques, which allow us to characterize and measure these changes. While these techniques are essential components for understanding synaptic plasticity, they each have their limitations and caveats to interpretation that must be considered.

This section will provide a brief background into how electrophysiological methods can be applied to measure changes in synaptic strength and make inferences about the mechanisms underlying these changes. In doing so, we look to make a strong case for the interpretation of the electrophysiological data presented in this thesis and clarify how these methods can shed crucial insights into the mechanistic functions of Wnt signaling during synaptic refinement.

1.3.1 The quantal hypothesis

Investigating how sensory experiences alter the structure and function of the brain can be a daunting task. However, the brain—like many complex systems—is comprised of more simple units, neurons, that are more easily understood and modeled. Neurons are discrete units in the brain that activate in an all-or-none fashion to transmit signals to other neurons distributed throughout the brain. But neurons, themselves, are still rather complex units that are comprised

of even more simple units, synapses, that facilitate the electrochemical communication between neurons. In this more refined sense, synapses can be thought of as the fundamental 'units' of neuronal computation. Thus, understanding the plasticity of the brain can be greatly simplified by understanding how the strength of individual synapses is altered.

To do this, however, requires a model for how neurotransmission takes place at a given synapse. The mechanism underlying synaptic transmission was discovered through the work of Katz and colleagues who observed that spontaneous miniature end-plate potentials (MEPPs) recorded from the frog neuromuscular junction (NMJ) were similar in shape and amplitude to minimally evoked end-plate potentials in the presence of high Mg^{2+} or low Ca^{2+}. This finding led them to propose the 'quantal hypothesis', which posits that synaptic neurotransmission is caused by the release of discrete packets (i.e., quanta) of neurotransmitters in an all-or-none fashion (Castillo & Katz, 1954; Fatt & Katz, 1952). From this hypothesis they devised a simple model for neurotransmission, stating that the measurement of a postsynaptic response (I) depends on the release probability (Pr) from a pool of releasable quanta (N) of defined quantal amplitude (Q). Therefore, the strength of an evoked postsynaptic response was formulated as:

$$I=QPrN$$

The value of this model is not only in its simplicity, but also in how these parameters correspond to distinct mechanisms that can be independently altered to change synaptic strength. For instance, Q is primarily a function of the number of available receptors at a postsynaptic site, while Pr corresponds to a presynaptic mechanism for how likely the firing of an action potential will result in the release of a synaptic vesicle. Thus, we can begin to not only understand how changes in synaptic strength are implemented, but where this change is likely to occur (either in the pre- or postsynaptic compartment).

However, while this model for synaptic transmission is useful, it can be overly simplistic to account for complex changes in synaptic strength. For one, the synapses of the peripheral nervous system (PNS), where this model originated, are much simpler than synapses of the central nervous system (CNS). The frog NMJ characteristically exhibits a high signal-to-noise ratio and quantal events that summate in a linear fashion (meaning each event carries approximately the same quantal amplitude). In contrast, measurements of synaptic parameters in the CNS are confounded by a low signal-to-noise ratio that renders small-amplitude quantal events difficult to detect, and the quantal amplitudes of these events vary considerably. Peripheral synaptic targets are also innervated by individual or a relatively low number of inputs, whereas neurons in the CNS receive numerous synaptic inputs arising from diverse brain regions, which impedes the attribution of spontaneous quantal events to a specific input.

Although this model for synaptic transmission is not without its limitations, it offers an intuitive explanatory framework for how changes in synaptic strength are implemented. I will now explore some of the techniques used that dissect the changes in these synaptic parameters, starting with methods examining spontaneous neurotransmission.

1.3.2 Methods for measuring spontaneous release

Unlike the high-fidelity transmission of electrical signals in computers, neurotransmission at synapses is far less predictable due to the spontaneous release of synaptic vesicles that occurs in the absence of neuronal firing. Because these spontaneous events are stochastic and each event corresponds to the release of a single synaptic vesicle, they offer valuable insight into the quantal parameters of synaptic transmission.

The recording of spontaneous currents is often done in the presence of TTX—referred to as miniature postsynaptic currents (mPSCs)—which prevents neuronal firing by blocking Na^+ channels, in order to prevent the synchronous release of synaptic vesicles. While leaving spontaneous neural activity intact—these are referred to as spontaneous postsynaptic currents (sPSCs)—can be useful to determine overall levels of synaptic drive, it also obfuscates the interpretation of these events as being quantal, since the synchronous release of vesicles from coactive inputs is not prevented (leading to an inflated estimate for quantal amplitude). Thus, it is often mPSCs, rather than sPSCs, that are used to evaluate quantal synaptic currents.

The recording of mPSCs relays two critical pieces of information. The first is the amplitude of quantal events (*Q*), which depends on the number of postsynaptic receptors. The second is the frequency of events, which is a function of both the probability of release, and the number of functional synaptic sites (*N*). While the probability of release is distinctly a presynaptic mechanism, the increase in the number of functional synapses can be caused by postsynaptic changes. This is especially true in developing systems, where it has been found that many nascent synapses are functionally 'silent' as they lack AMPARs and contain only NMDARs, which open only when the neuron is depolarized by coactive inputs (Isaac et al., 1995, Liao et al., 1995). Therefore, the unsilencing of immature synapses through postsynaptic AMPAR recruitment presents another possible interpretation for changes in mPSC frequency.

Thus, while the interpretation of changes in mEPSC amplitude is unambiguous, changes in event frequency are not as clear and require further experimentation to elucidate whether the underlying mechanism is due to a pre- or postsynaptic change.

1.3.3 Methods for measuring evoked release

Methods that rely on evoked synaptic release—either through sensory stimulation, electrical stimulation, optogenetics or chemogenetics—can give further clarity into the mechanisms underlying changes in synaptic strength. One advantage of using evoked responses to study changes in synaptic strength is that the experimenter has more precise control over which inputs are stimulated and the level at which they are stimulated. As mentioned previously, inputs onto CNS neurons often arise from different areas, which cannot be distinguished when only spontaneous events are recorded. Thus, the precise activation of inputs allows one to infer changes to a particular synaptic input rather than synapses more broadly.

An example of where this becomes useful is to detect changes in the probability of release of specific inputs by measuring the paired-pulse ratio (PPR). When an action potential travels down the axon to depolarize presynaptic terminals, this results in the activation of voltage-dependent Ca^{2+} channels to trigger the mobilization and fusion of neurotransmitter vesicles. When two pulses are paired in quick succession (typically 20–100 ms), residual Ca^{2+} left over from the first stimulus will transiently increase the presynaptic release probability (*Pr*) upon the second stimulus, termed short-term plasticity (STP) (Zucker & Regehr, 2002). The relative peak amplitude of the first and second pulse, known as the paired-pulse ratio (PPR), therefore directly relates to *Pr*. If the presynaptic terminal has a high *Pr*, then the readily releasable pool of synaptic vesicles will be mostly depleted from the first pulse, and so less vesicles will be available for release during the second pulse. This would have the effect of decreasing the PPR. In contrast, synapses with a low *Pr* will have less synaptic vesicles depleted by the first pulse, so they will have more available to release upon the second pulse when presynaptic STP is in effect. This would cause the PPR to increase. Consequently, changes in

PPR have been interpreted to reflect presynaptic changes in *Pr*, however there are some important caveats to this interpretation.

There are also various postsynaptic mechanisms of STP which involve AMPAR desensitization (Constals et al., 2015; Heine et al., 2008), AMPAR surface diffusion (Opazo et al., 2010) and AMPAR permeability (Burnashev et al., 1992). Of these mechanisms for postsynaptic STP, changes in AMPAR permeability appear to play an influential role in the development of retinotectal synapses. In this system, calcium-permeable AMPA receptors (CP-AMPARs) mediate fast excitatory synaptic transmission but are tonically blocked by intracellular polyamines (Anggono & Huganir, 2012). However, this polyamine block can be relieved by repetitive stimulation to increase the permeability of CP-AMPARs, resulting in a postsynaptic form of STP that transiently increases the amplitude of excitatory currents (Toth et al., 2000). In the retinotectal system of *Xenopus laevis*, it was shown that a 4-hour period of visual stimulation upregulates polyamine synthesis, resulting in an increased PPR (Aizenman et al., 2003). Thus, changes in PPR are not always expressed by a purely presynaptic mechanism and methods that are able to better isolate postsynaptic changes can add some clarity to this interpretation.

One approach for isolating postsynaptic changes relies on comparing the evoked amplitude of the AMPAR-mediated and NMDAR-mediated currents—termed the AMPA/NMDA ratio. This method takes advantage of differences in voltage-dependence and decay kinetics between these receptor subtypes. Glutamate binds to both AMPA and NMDA receptors, with low and high affinity, respectively (Lester & Jahr, 1992; Patneau & Mayer, 1990). However, NMDARs do not flux ionic current when the membrane is near typical resting potential (-60 mV) due to strong affinity for Mg^{2+} within the receptor ionophore, effectively

blocking cationic movement upon glutamate receptor binding. In contrast, neuronal depolarization reveals an outward mixed synaptic current, consisting of both AMPAR-mediated and NMDAR-mediated components. Given the different time constants of NMDAR and AMPAR currents, the relative contribution of each receptor subtype can be readily dissected. While the initial component of the evoked EPSC shows a fast rise-time, including both NMDAR and AMPAR components, the rapid decay of AMPAR responses reveals a pure NMDAR-mediated current by >50 ms post-stimulus. This method of measuring AMPAR currents at hyperpolarized membrane potentials (typically -60 to -70 mV) and NMDAR currents around 50 ms post-stimulation at depolarized postsynaptic membrane potentials (typically +40 mV) allows for electrophysiological delineation of the glutamate receptor subtypes in the absence of pharmacological antagonists and is a fast and efficient measure of plastic changes in the composition of synaptic receptors.

The AMPA/NMDA ratio is highly useful as a measure of synaptic strength, as well as the maturity of nascent synapses. Whereas NMDAR levels remain relatively constant (Wu et al., 1996), AMPARs are dynamically regulated and trafficked to synapses which is essential for the expression of LTP at many synapses (Malenka & Bear, 2004). Thus, a higher AMPA/NMDA ratio depicts an increase in the strength of synapses. Furthermore, an elevated AMPA/NMDA ratio can indicate synaptic maturity in developing systems. As mentioned previously, many nascent synapses in developing circuits contain only NMDARs, rendering them functionally 'silent'. The conversion of these silent, immature synapses requires the recruitment of AMPARs, thereby increasing both the number of functional synapses and the ratio of AMPA/NMDA responses. Indeed, studies in the retinotectal system of *Xenopus laevis* tadpoles show a developmental gradient of AMPA/NMDA ratios which are higher in the rostrolateral tectum,

where there are more mature neurons, and lower in the caudomedial tectum where neurons are more immature (Wu et al. 1996). Thus, the AMPA/NMDA ratio serves as an essential indicator for postsynaptic maturity during retinotectal development.

Taken together, each of these electrophysiology methods offers unique insight into the mechanistic changes in synaptic strength. mPSCs provide insight into quantal parameters *Q* but fails to distinguish between changes in *Pr* and *N*. PPR is likely to reflect changes in *Pr* and can be further supported by ruling out postsynaptic changes by measuring the AMPA/NMDA ratio. It is through a combination of these methods that we attempt to interpret the mechanistic functions of pathways like Wnt signaling in altering the function of refining synapses.

1.4 Project Rationale

The retinotectal system constitutes a highly tractable model for elucidating the general mechanisms and principles of neural circuit development. The topographic organization of this circuit is achieved by a confluence of hardwired and experience-dependent mechanisms to establish this precise synaptic connectivity. Although visual experience plays a significant role in the refinement of the retinotectal circuit, we have yet to discover the mechanisms by which patterned activity detected at postsynaptic sites modulates the formation and maintenance of functional presynaptic sites. The most likely mechanism for mediating trans-synaptic Hebbian plasticity is through the transmission of secreted retrograde signals that can convey activity-dependent information. Various candidates for activity-dependent retrograde signaling have been investigated, however the candidates studied thus far do not appear to account for this mechanistic function.

One promising candidate for activity-dependent retrograde signaling are the widely conserved intercellular signaling molecules Wnts. Wnts are implicated in a wide variety of synaptic functions including axon guidance, dendritogenesis, and synaptic plasticity. During retinotectal development, the canonical pathway Wnt ligand, Wnt3A has been implicated in modulating receptive-field plasticity, but the mechanisms by which it does so remain to be understood. The purpose of this thesis project is to: 1) characterize the effects of Wnt signaling on synaptic physiology and neuronal morphology and 2) elucidate the mechanisms by which Wnt signaling may be contributing to pre- and/or postsynaptic remodeling. In doing so, we seek to advance our understanding of how Wnt signaling influences circuit development, and more broadly, our understanding of the molecular principles by which sensory experiences shapes the structure and function of the developing brain.

Chapter 2: Methodology

2.1 Animals

All experiments were approved by the Montreal Neurological Institute Animal Care Committee in accordance with Canadian Council on Animal Care guidelines. Albino *Xenopus laevis* tadpoles (RRID:NXR_0.0082) were produced from our in-house breeding colony. Female frogs were primed with a 50 IU injection of pregnant mare serum gonadotropin (PMSG, Prospec). Three days later human chorionic gonadotropin (HCG, Sigma) was injected into a male (150 IU) and the primed female (400IU), after which the pair was placed together in an isolated tank for mating. Eggs were collected the following day and kept in standard 0.1x Modified Barth's Saline-H (MBSH).

For *in vitro* fertilizations, eggs from primed females were collected and fertilized using thawed sperm aliquots from transgenic frogs (Xla.Tg(WntREs:dEGFP); NXR_0064, Xenopus National Resource, Woods Hole) harboring a pbin7Lef-dEGFP construct (generous gift from the Vleminck lab) (Tran & Vleminckx, 2014) to generate animals with a reporter for active canonical Wnt signaling.

2.2 Immunohistochemistry

To assess whether Wnt signaling was active in the OT during retinotopic development, pbin7Lef-dEGFP transgenic tadpoles were immunostained for EGFP fluorescence at stages 45 and 48 (Nieuwkoop & Faber, 1994). Tadpoles were anesthetized by immersion in 0.02% tricaine

mesylate (MS-222, Sigma) in MBSH and fixed in 4% paraformaldehyde (Cedarlane) in 0.1M phosphate buffered saline (PBS) for 1 hr and then fixed in 100% methanol overnight. Animals were cryoprotected by consecutive overnight incubations in 15% and 25% fish gelatin (Norland) with 15% sucrose. On the day of sectioning, tadpoles were embedded in 20% fish gelatin/15% sucrose. Tadpoles were cryosectioned in the horizontal plane into 15 μm thick sections on a cryostat and mounted onto Superfrost-plus slides (Fisher). Slides were washed with 1% sodium dodecyl sulfate (Bioshop) for 5 min, followed by incubations with blocking solution—5% normal goat serum (Sigma) and 1% bovine serum albumin (Fisher)—and PBS.

The primary antibody used to label GFP+ cells was a chick monoclonal anti-GFP (Abcam) at a ratio of 1:1000 and visualized using a goat anti-chick Alexa-488 fluorescence-conjugated secondary antibody (1:200, Invitrogen). Stained sections were mounted using AquaPolyMount medium (Polysciences). Images were acquired with a Zeiss LSM 710 inverted confocal microscope.

2.3 Electroporation

Electroporations were performed as described (Ruthazer et al., 2013a, 2013b, 2013c). Albino *Xenopus laevis* tadpoles were anaesthetized by immersion in 0.02% MS-222 (Sigma) diluted in 0.1X MBSH and placed on a Kimwipe under a dissection microscope.

For the RGC axon imaging experiments, retinal electroporations were performed by pressure injecting a small volume of plasmid (1.5 μg/μl) encoding XDsh-ΔPDZ-GFP (generous gift from Randall Moon, Addgene plasmid #16786) and mCy-RFP at a ratio of 3:1, respectively,

into the vitreous humor of the eye in stage 40-42 tadpoles. A pair of custom-made platinum plate electrodes, connected to an electrical stimulator (SD 9, Grass Instruments), was placed on both sides of the eye, and 4-6 pulses (30-40 V intensity, 1.6 ms duration) were delivered unidirectionally to target ventral RGCs. A 3μF capacitor was connected in parallel to the electrodes to produce an exponential waveform.

The bulk labelling of tectal neurons for the electrophysiology experiments was performed by injecting plasmids encoding pEGFP-N1 (1 μg/μl, Clonetech) or a bidirectional BICS2-XWnt3a-GFP (1 μg/μl, made in-house by Anne Schohl) construct into the tectal ventricle of stage 43-45 animals and pulses were administered 3-5 times in both directions.

For studying tectal morphology dynamics, CRE-Mediated Single-Cell Labeling by Electroporation (CREMSCLE) (Schohl et al., 2020) was used to label isolated tectal neurons. Briefly, this method relies on the co-expression of separate Cre-recombinase and a Cre-dependent fluorophore plasmids at disparate ratios to ensure a relatively low rate of co-transfection. pCAG-Cre and pCALNL-GFP (both plasmids a generous gift from Connie Cepko, Addgene plasmid #'s 13775, 13770) were electroporated at a ratio of 1:4000 (0.25 ng/μl : 1 μg/μl) along with an additional pCS-XWnt3A plasmid (1 μg/μl, made in-house by Anne Schohl) for the experimental condition at a ratio of 1:4000:4000 Cre:GFP:XWnt3A. A relatively high concentration of XWnt3A plasmid compared to pCAG-Cre was used to ensure the co-expression of these constructs in cre-expressing GFP-labeled tectal neurons.

To measure the number and density of PSD95 puncta in tectal dendrites, bulk tectal electroporations were performed by injecting a plasmid solution containing BICS2-XWnt3a-mCherry (made in-house by Anne Schohl) and PSD95-GFP (a gift from Dr. Hollis Cline) at a

total concentration of 2µg/µl, and a ratio of 1:1. For the control condition a plasmid solution containing pcDNA-mCherry and pPSD95-GFP was used, also at a total plasmid concentration of 2µg/µl, and a ratio of 1:1. Electroporations were performed in stage 43-44 animals and pulses were administered 1-2 times in both directions to optimize the sparse labeling of tectal neurons.

2.4 Electrophysiology

Tectal whole-cell patch-clamp recordings were made in the isolated intact brain of stage 45 – 48 albino *Xenopus laevis* tadpoles. Tectal preparations were acquired by anaesthetizing tadpoles in 0.02% MS-222 and placing them in a chilled extracellular recording solution (in mM – 115 NaCl, 2 KCl, 3 CaCl2, 3 MgCl2, 5 HEPES, and 10 glucose, pH 7.20, 250 mOsm). A fine-pointed scalpel was used to make an incision along the dorsal midline of the OT to expose the brain tissue, allowing for it to be dissected out and pinned to a Sylgard block in a recording chamber filled with room temperature extracellular recording solution. Patch-clamp electrophysiology was performed by backfilling a recording pipette (6-12 MΩ, Sutter Instruments) with cold cesium-containing internal solution (in mM – 90 CsMeSO4, 20 HEPES, 20 tetraethylammonium, 10 EGTA, 5 MgCl2, 2 ATP, 0.3 GTP, pH 7.20, 250 mOsm). To gain access to tectal neurons, part of the ventricular membrane was carefully removed using a broken micropipette. Individual tectal neurons were visualized using an Olympus BX51 upright microscope with a 60x (0.9 NA) water-immersion objective and a CCD camera (Sony XC-75). Recordings were obtained using an Axopatch 200B amplifier (Molecular Devices). Signals were digitized using the Digidata 1550 (Molecular Devices), sampled at 10 kHz, and filtered at 2 kHz. Recordings were collected using pClamp 10.4 software (Molecular Devices). Series resistance

was monitored throughout the duration of an experiment and recordings were discarded if there was more than a 20% change in series resistance.

mEPSCs were recorded from tectal neurons held at -70mV in the presence of the GABA-A antagonist, picrotoxin (PTX, Abcam) (100 μM), and tetrodotoxin citrate (TTX, Alomone Labs) (1 μM), to block inhibitory postsynaptic currents and neuronal firing, respectively. MiniAnalysis (Synaptosoft) and Clampfit 10.2 (Molecular Devices) software was used to detect and measure mEPSC events in a semi-automated fashion.

Electrical stimuli were generated with an ISO-flex stimulus isolation unit (AMPI), delivered to the optic chiasm with a custom bent 25 mm cluster electrode (FHC). Current pulses (100 μs) were given at various stimulus intensities in the presence of PTX (100 μM) to isolate the excitatory component of evoked responses. AMPAR/NMDAR ratios were measured by holding cells at -70 mV or +40 mV to determine AMPAR or NMDAR amplitudes, respectively. The AMPAR amplitude was calculated by measuring the peak of the response, whereas NMDAR amplitude was calculated by taking a 5 ms average of the response amplitude 50 ms after excitation. Paired-pulse recordings were performed at -70 mV using an interstimulus interval of 50 ms, and ratios were calculated by dividing the peak amplitude of the second response by the peak amplitude of the first. PPR and AMPA/NMDA ratio measurements were done using Clampfit 10.2 software (Molecular Devices)

2.5 Two-photon imaging

In vivo two-photon imaging was performed using a confocal microscope custom-built for multiphoton imaging with a 60x water-immersion objective (1.0 NA). Excitation light was produced by a Maitai-BB Ti:Sapphire or an InSight X3 femtosecond pulsed IR laser (Spectra Physics), and z-series optical sections were collected at 1 μm intervals using Fluoview software (version 5.0). Emission in the green and red spectrum was done using a 525/50 and 630/92 bandpass filter, respectively.

At 24 - 48 h after electroporation, animals were screened for the expression of labelled constructs in RGCs or tectal neurons. For daily imaging, animals were anesthetized in MS-222 (0.02% in 0.1% MBSH) and imaged once a day for four consecutive days and placed in a custom-made Sylgard chamber that was fit to the tadpole's body.

For dynamics imaging, animals were immobilized by immersion in 2 mM pancuronium dibromide (Tocris) and embedded in low melting point agarose on a small Petri dish, with images collected every 10 min for up to 1 h. Imaging of mCy-RFP-expressing cells was performed using excitation light of 990nm while GFP imaging was done using 910nm. Two-photon image acquisition took less than 10 min, after which the animals were returned to an isolated well that contained freshly prepared MBSH.

For the imaging of tectal morphology dynamics, 2-photon images were captured using 910 nm excitation immediately afterwards animals were subjected to a period of 4 h in darkness, followed by 4 h of short-term enhanced visual experience (STVE) consisting of a 3x4 grid of LEDs that move unidirectionally at a rate of 0.3Hz (Sin et al., 2002).

For the imaging of PSD95-GFP puncta in tectal dendrites, images were captured exciting at 990nm using both red and green channels in stage 47-48 tadpoles.

2.6 Image analysis

All z-stack images collected by the two-photon were denoised using CANDLE software (Coupé et al. 2012). For the experiment examining axon branch dynamics, cell tracing was done using Dynamo software (Kurt Haas lab, written in MATLAB) to quantify branch losses and additions (Hossain et al., 2012). For the daily imaging of axons and dynamics imaging of tectal neurons, morphological reconstructions were done using Imaris 6.4.2 software (Bitplane).

For the counting of PSD95-GFP puncta on tectal dendrites, morphological reconstructions were done using the SNT plugin through the Fiji distribution of ImageJ (Arshadi et al., 2021). In some cases, the imaged field had more than one dendritic arbor present, but only dendritic arbors that expressed both GFP and RFP were selected for reconstruction. The reconstructions were used to create a binary mask of the dendrite using the 'Fill' method in SNT (manual threshold set between 0.03 and 0.05), which was used to isolate the dendritic segments within the GFP channel. The GFP channel containing only the isolated dendritic segments was first processed using the 'Subtract Background' method with a rolling ball radius of 50 pixels and then collapsed into a 2D z-projection of the maximum pixel intensity. To enhance the punctate regions along the dendrite, this image was further processed in Fiji using a difference of Gaussian method by applying a 2D Gaussian blur of a 1- and 2-pixel radius to two separate copies of the image, and then subtracting these images using the 'Image Calculator' function. Thresholding was consistently applied to maximize the detection of puncta and 'watershedding'

was performed to separate overlapping puncta. Puncta were then counted using the 'Particle Analyzer' function in Fiji, with the circularity threshold set to 0.6 and the particle size threshold set between 0.1 and 3 μm^2.

2.7 Quantification and statistical analysis

All data are expressed as mean (± SEM), and n values refer to the number of cells. Results were considered statistically significant when $p < 0.05$. Statistical analysis was performed using GraphPad Prism 6.

Chapter 3: Results

3.1 Region-specific activation of canonical Wnt signaling during retinotopic refinement

To verify whether there is active Wnt signaling in the tectum during retinotopic refinement, we decided to use a previously validated transgenic Xenopus line that reports canonical Wnt signaling activity (Tran & Vleminckx, 2014). The transgenic reporter construct, pbin7Lef-dEGFP, harbors a TCF/LEF promoter sequence – the promoter used for transcription via the canonical Wnt signaling pathway – directly upstream of dEGFP2. dEGFP2 is a destabilized GFP reporter that maintains fluorescence activity for approximately 2 hours, ideal for tracking time sensitive Wnt activity regulation (Li et al., 1998). To validate the otherwise dim fluorescence signal, immunohistochemistry was performed with anti-EGFP antibodies at stages 45 and 48 which span a dynamic phase of retinotopic refinement.

EGFP fluorescence is clearly observed at stage 45 and 48 with the degree of fluorescence higher in the more immature, stage 45 animals, suggestive of a developmental regulation of canonical Wnt signaling activation (**Fig. 3.1A**). Nonetheless, both stages displayed a consistent staining pattern of a dorsal-ventral decreasing Wnt activity gradient, in agreement with previous *in situ* studies for XWnt3A expression (Lim et al., 2010; Wolda et al., 1993). Interestingly, the staining pattern appears to be quite sparse, suggesting that Wnt signaling activity may not only be regulated regionally and temporally, but in a cell-type specific manner as well. Conversely, canonical Wnt signaling activity was not observed to the same degree in the RGC layer of the retina in stage 48 animals (**Fig. 3.1B**). This finding, along with the fact that Xfz5 (Sumanas & Ekker, 2001) and Xfz2 (Rodriguez et al., 2005) receptors display a retina-specific expression,

support the notion that Wnts likely act on incoming RGCs via transcription-independent pathways.

To verify whether the reporter construct was responsive to pharmacological induction of canonical Wnt signaling, stage 38 animals were reared in 10 mM LiCl, which is a potent activator of the canonical Wnt signaling pathway. Brightfield fluorescence was adequately sensitive to validate the responsiveness of the Wnt reporter to increased Wnt signaling, evident by a clear and robust phenotype observed in dEGFP patterning in the tail (**Fig. 3.1C**). Compared to controls, LiCl treatment drastically increases the number of EGFP positive cells in the tail region and disrupts the parallel arrangement of EGFP-positive cells observed in control animals.

Taken together, our observations recapitulate the findings from other studies showing that Wnt signaling exhibits region-specific activation in the tectum during retinotopic refinement, and that the primary source of Wnts are postsynaptic tectal neurons. Furthermore, the use of a reporter for canonical Wnt signaling activation, as opposed to just looking at XWnt3a expression patterns, indicates that Wnts may have functional consequences on postsynaptic transcription and morphology beyond their retrograde actions on presynaptic axons.

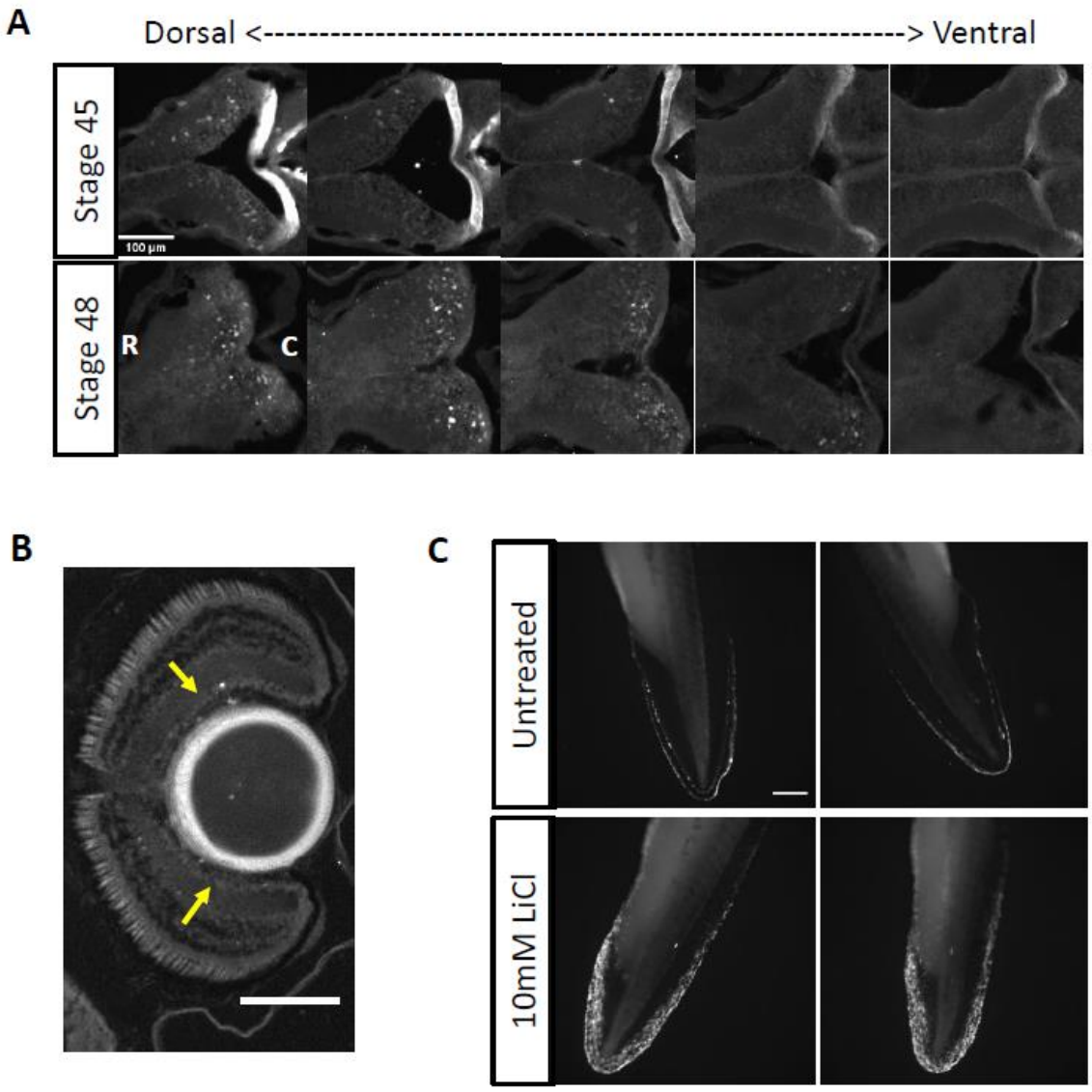

Figure 3.1: The canonical Wnt pathway is active in the optic tectum during retinotectal development. (A) Immunostaining for EGFP was performed on stage 45 and 48 transgenic tadpoles expressing pbin7Lef-dGFP which reports canonical Wnt activation. Tadpole sections from the OT and midbrain/hindbrain are displayed from dorsal (leftmost section) to ventral (rightmost section). All sections are similarly oriented along the rostral [R] and caudal [C] axis (shown in the bottom, leftmost image). Each section has a thickness of 15µm (B) Immunostaining for EGFP expression in the eye of stage 48 tadpoles. The arrows (yellow) point to the RGC cell-body layer in the retina which appear to lack a strong signal. The scale bar is 100µm (C) Treatment of stage 38 animals with the potent canonical pathway activator LiCl (10mM) results in a striking phenotype in the tail of these tadpoles, increasing both the number of EGFP positive cells and their typified parallel arrangement. The scale bar is 100µm.

3.2 Overexpression of Wnt3A promotes the functional development of retinotectal synapses

Since canonical Wnt signaling is active during retinotopic refinement, we assessed whether Wnt signaling modulates synaptic physiology by overexpressing Wnt3A in tectal neurons. To overexpress Wnt3A we performed tectal electroporations using a bidirectional XWnt3A-BICS-GFP construct or EGFP as a control in stage 43-45 animals. This resulted in sparse transfection of tectal neurons which expressed GFP. GFP-positive neurons were subjected to patch-clamp electrophysiology 3-5 days after electroporation (**Fig. 3.2A**).

To determine whether synapses were affected by overexpression of Wnt3A, AMPA mEPSCs were recorded from stage 48 animals (**Fig. 3.2B-D**). Wnt3A overexpression resulted in a significant increase in mEPSC frequency compared to controls (**Fig. 3.2C**) with no change in amplitude (**Fig. 3.2D**). This result could be explained by a Wnt3A-mediated increase in presynaptic release probability or an increase in the number of functional synapses. To determine if the increase in mEPSC frequency caused by Wnt3A overexpression was due to an enhancement of presynaptic release, paired-pulse ratio (PPR) recordings were performed. We found that overexpression of Wnt3A did not alter PPR compared to EGFP-expressing controls (**Fig. 3.2E**), suggesting that the postsynaptic expression of Wnt3A did not modulate evoked presynaptic release in a retrograde manner. Furthermore, to assess the influence of Wnt3A on synaptic maturation, AMPAR/NMDAR ratios of evoked responses were measured, showing significantly increased ratios in Wnt3A-expressing neurons relative to controls (**Fig. 3.2F**). This suggests that Wnt3A expression promotes the functional maturation of synaptic inputs. The complementary findings that Wnt3A overexpression increases mEPSC frequency without

changing mEPSC amplitude or PPR could be explained by Wnt3A promoting increased synapse formation or by functional maturation of synaptic inputs. The observation of increased AMPA/NMDA ratios is most consistent with the functional maturation of synaptic inputs by unsilencing of NMDAR-only “silent” synapses. Taken together, these data suggest that Wnt3A contributes to the functional maturation of developing synapses through a postsynaptic cell-autonomous mechanism of action.

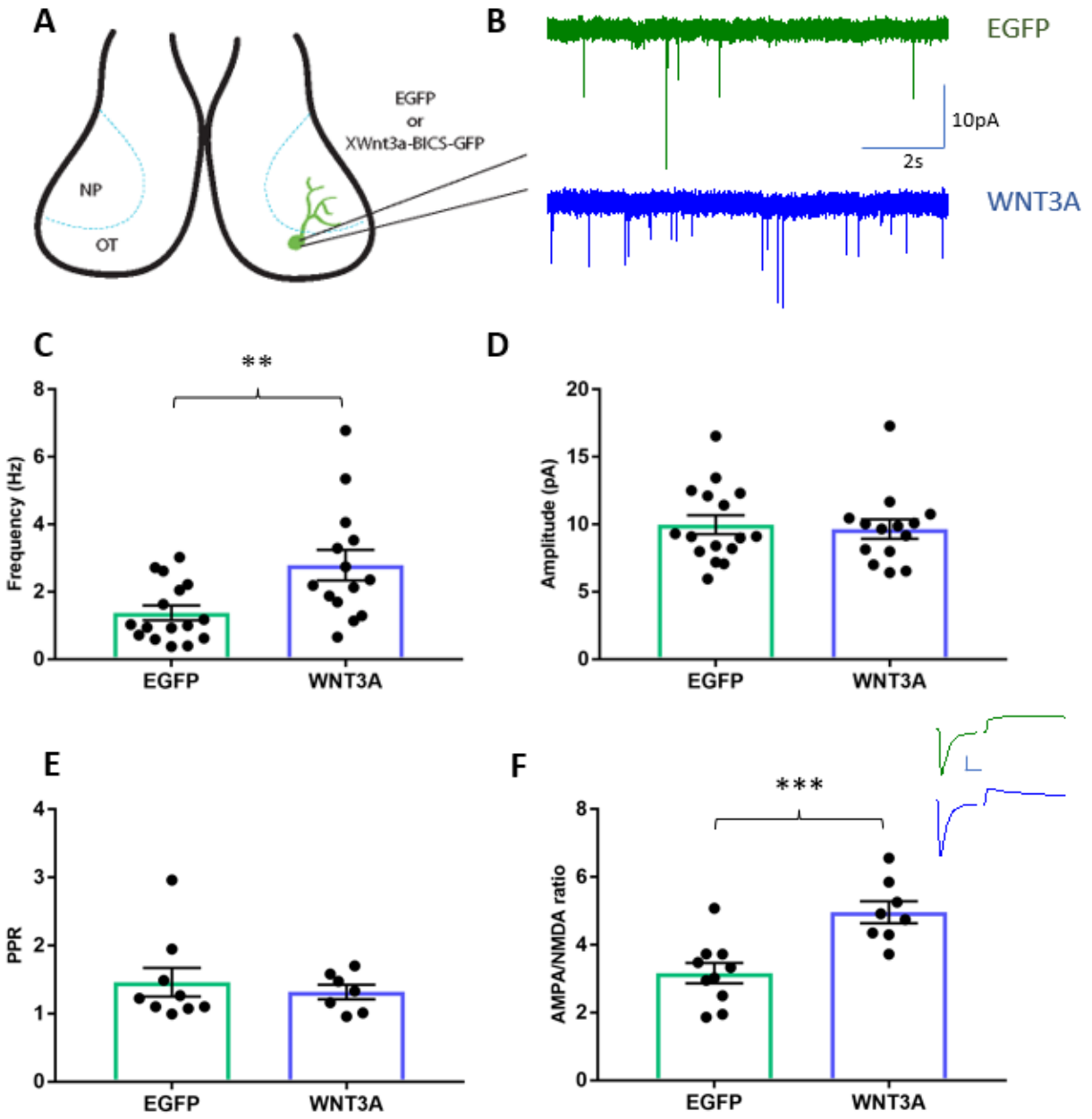

Figure 3.2: Overexpression of Wnt3A promotes the functional development of retinotectal synapses (A) Schematic depicting the method for performing patch-clamp electrophysiology of GFP labelled tectal neurons. (B) mEPSC traces of tectal neurons expressing EGFP (green) or Wnt3A (blue). mEPSC events are viewed as downward deflections from the baseline holding potential. (C-D) Group data showing the frequency (C) and amplitude (D) of mEPSCs. (C) mEPSC frequency is significantly increased in Wnt3A neurons (2.8 ± 0.45, n=14) compared to controls (1.4 ± 0.22, n=16) (**P=0.0068, two-tailed t-test). (E) Group comparisons of paired-pulse ratios were calculated as the second peak amplitude divided by the first peak amplitude (Peak2/Peak1). The paired-pulse stimulation interval is 50ms. (F) AMPAR/NMDAR ratios were calculated as the peak of the AMPAR current, holding at -70mV, divided by a 5ms average of the NMDAR amplitude at 50ms after the stimulus, holding at +40mV. The scale bar is 50pA by 10ms. AMPAR/NMDAR ratio is significantly increased in Wnt3A neurons (5.0 ± 0.32, n=8) compared to controls (3.2 ± 0.30, n=10) (***P=0.0009, two-tailed t-test). Data (C-F) are presented as mean ± SEM.

3.3 Wnt3A overexpression increases the density of synaptic puncta in tectal dendrites

Since our physiology data suggests that Wnt3A may be promoting the formation or maturation of synapses, we looked to measure the number and density of postsynaptic puncta in tectal neurons overexpressing Wnt. In stage 43-44 tadpoles, we performed tectal co-electroporations of PSD95-GFP to label postsynaptic puncta and BICS2-XWnt3A-mCherry or mCherry to trace tectal dendrite morphology. Images of isolated dendritic arbors were captured 3 days later, and morphological reconstructions were made to define regions of interest for PSD95 puncta detection along the arbor (**Fig. 3.3A**).

While Wnt3A-expressing neurons showed no significant difference in dendritic branch length (**Fig 3.3B**) or the total number of puncta (**Fig 3.3C**), Wnt3A-expressing neurons did show a significant increase in the density of synaptic puncta (number per dendritic branch length) compared to controls (**Fig 3.3D**). This increase in synapse density is likely to reflect the role of Wnt3A in increasing functional synapse number by increasing the likelihood that a postsynaptic site along the dendritic arbor is stabilized and retained. It is interesting to note that the NMDAR co-agonist D-serine has also been shown to increase PSD95 punctum density (Chorghay et al., 2021) and has been implicated in the maturation and unsilencing of synapses (Van Horn et al., 2017). Thus, our finding that Wnt3A expression increases synapse density on tectal dendrites lends further support to the notion that Wnt3A promotes synaptic maturation through a cell-autonomous postsynaptic mechanism.

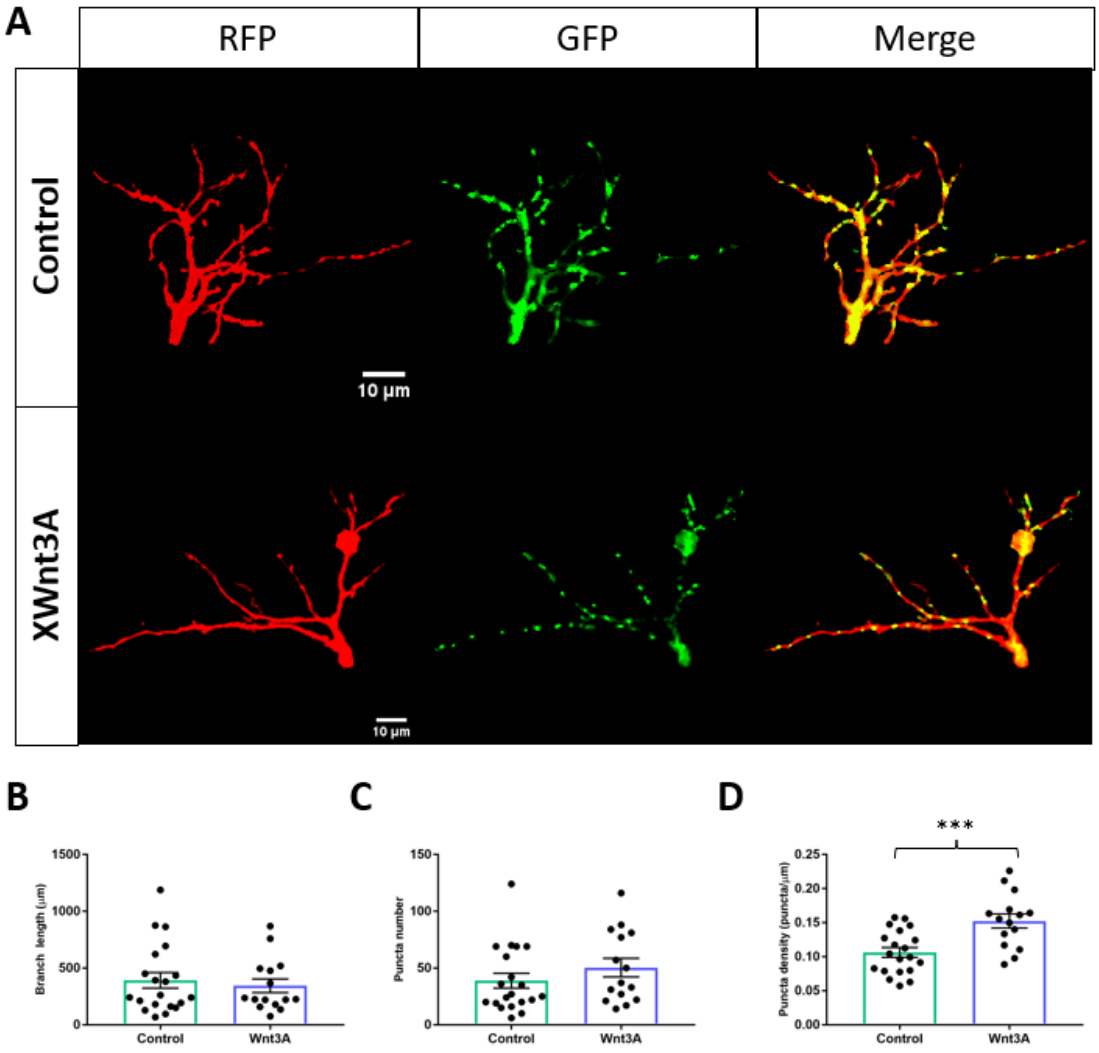

Figure 3.3: Wnt3A overexpression increases PSD95 puncta density in tectal dendrite arbors (A) Representative micrographs of tectal dendrite arbors that were selected for morphological reconstruction. (B) Comparison of the average dendritic branch length per cell between control and Wnt3A expressing neurons. (C) Comparison of the average number of PSD95 puncta per cell between control and Wnt3A expressing neurons. (D) Group data showing a significant increase in puncta density for Wnt3A expressing dendrites (0.1525 ± 0.0103, n=15) compared to controls (0.1062 ± 0.007, n=20) (***P=0.0006, two-tailed t-test). Data (B-D) are presented as mean ± SEM.

3.4 Wnt3A overexpression enhances sensory-dependent dendritic branch growth

Having provided evidence that Wnt3A overexpression promotes the maturation of developing synapses, we next wanted to see if Wnt3A overexpression regulates tectal dendrite morphology in an activity-dependent manner. To do this we used a previously published methodology to assess the short-term influence of enhanced visual experience on dendritic growth (He et al., 2016). Animals were subjected to a 4-hour period of darkness followed by 4 hours of short-term enhanced visual experience (STVE), which consists of a housing chamber with a 3x4 grid of LED lights that repeatedly flash to simulate unidirectional motion (Shen et al., 2014). Two-photon z-stack images were taken before and after each 4-hour conditioning period, comprising a total of three timepoints (**Fig. 3.4A**). Isolated tectal neurons were labelled using CRE-Mediated Single-Cell Labeling by Electroporation (CREMSCLE) (Schohl et al., 2020), and Wnt3A was co-expressed to evaluate its impact on dendritic branch growth and dynamics.

We found that the overexpression of Wnt3A significantly increased the length of tectal branches over the total 8-hour imaging period relative to controls (**Fig. 3.4B**). On the other hand, changes in branch number were not significantly different between Wnt3A dendrites and controls (**Fig. 3.4C**). When comparing the growth of branches during 4 hours of darkness versus 4 hours of STVE, we found that neurons expressing Wnt3A grew significantly longer branches during visual experience, but not during darkness (**Fig. 3.4D**). Conversely, overexpression of Wnt3A did not change the number of branches relative to controls in either darkness or STVE (**Fig. 3.4E**).

Furthermore, presenting this data as a scatterplot to compare the growth of individual tectal neurons during darkness (x-axis) versus STVE (y-axis), reveals a clustering of Wnt3A-expressing neurons relative to controls (**Fig. 3.4F-G**). Comparing branch length, Wnt3A-expressing neurons display a dispersion to the upper-right quadrant (**Fig. 3.4F**), suggesting their magnitude of growth is greater than controls but with no strong preference towards growth in conditions of darkness or visual stimulation. Conversely, comparing the changes in total dendritic branch number reveals a dispersion of Wnt3A-expressing cells towards the upper y-axis relative to controls (**Fig. 3.4G**), suggesting that there may be a preference for dendrites expressing Wnt3A to increase their number of branches under conditions of visual stimulation.

This data indicates that Wnt3A overexpression promotes dendrite branch elongation, further supporting a postsynaptic function of Wnt signaling during retinotectal development. Importantly, the dynamics of tectal dendritic arbors is thought to reflect synaptic stability, as the formation of stable synapses can facilitate the extensional growth of the arbor (Cline & Haas, 2008). Thus, our observation that Wnt3A promotes the enhanced growth of tectal dendrites can be reconciled by a mechanism that involves the functional maturation of postsynaptic contacts. Moreover, the difference in dendritic branch length between Wnt3A and control neurons during visual stimulation suggests that Wnts may have an influence on activity-dependent processes that regulate growth in tectal dendrites. Taken together, our data implicate Wnt3A in promoting the activity-dependent maturation of retinotectal synapses and strongly suggest a novel role for postsynaptic Wnt signaling in retinotopic development.

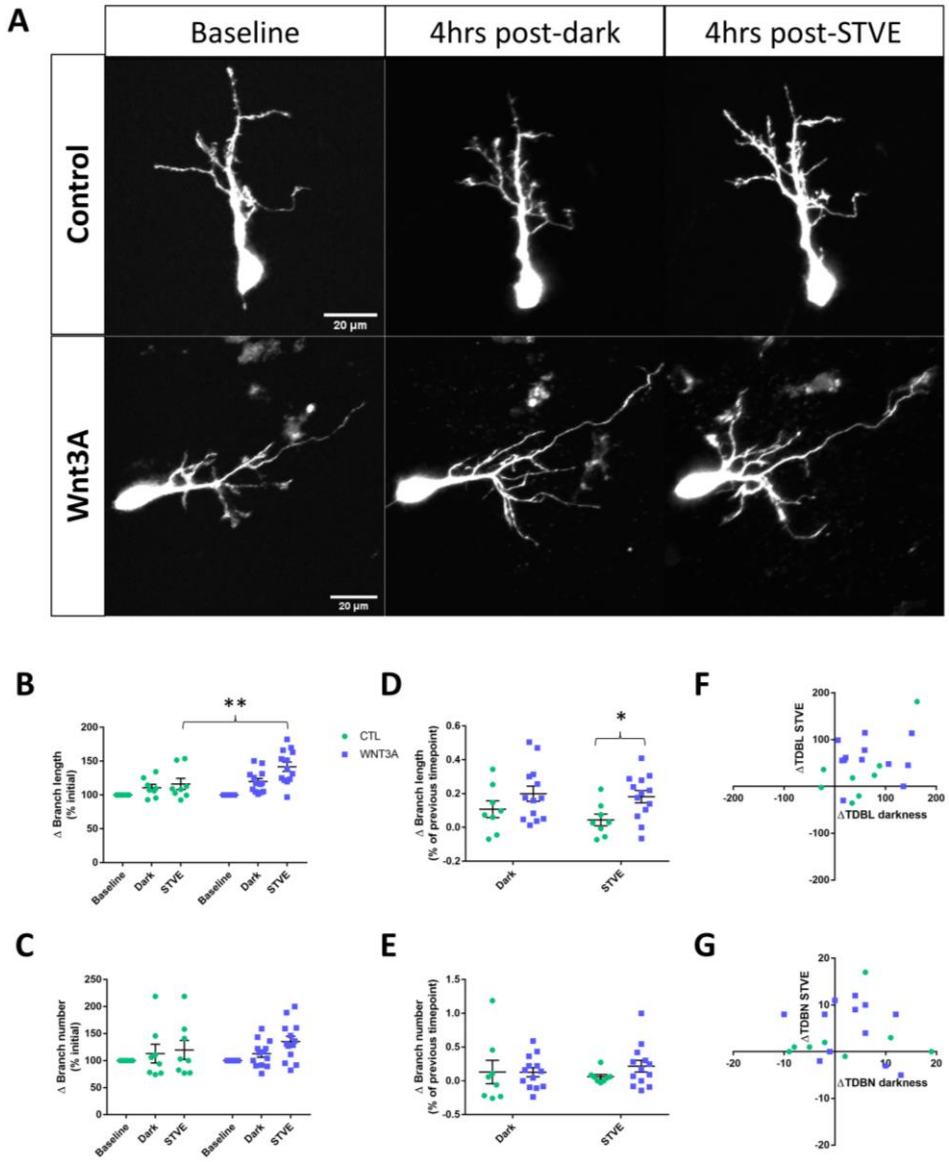

Figure 3.4: Wnt3A overexpression enhances sensory dependent dendrite branch growth. (A) Representative micrographs of control and Wnt3A-expressing tectal dendrites at baseline, 4 hours post-darkness, and 4 hours post STVE. Comparison of changes in tectal dendritic branch length (B) and branch number (C) during darkness and STVE, normalized to the branch length and number at baseline, respectively. (B) Overexpression of Wnt3A (n=13) increases normalized branch length compared to control (EGFP) dendrites (n=8) (interaction, *P=0.014, 2way-ANOVA; Holms-Sidak multiple comparisons test – STVE **P=0.0035). (D) Breakdown of changes in total dendritic branch length normalized to the branch length at the previous timepoint after darkness and STVE, showing Wnt3A dendrites grow longer branches compared to controls during STVE (CTL: 0.044 ± 0.035, Wnt3A: 0.18 ± 0.037, *P=0.020, two-tailed t-test) but not dark-rearing (E) Breakdown of changes in total dendritic branch number during dark-rearing and STVE, normalized to the branch number at the precious timepoint. Scatterplots showing the changes in total dendritic branch length (TDBL) (F) and total dendritic branch number (TDBN) (G) of individual tectal neurons during darkness (x-axis) and STVE (y-axis). Changes in TDBL are displayed as the change in total arbor length as compared to the previous timepoint (µm) and changes in TDBN are displayed as the absolute change in branch number relative to the previous timepoint. Data (A-D) are presented as mean ± SEM.

3.5 Wnt signaling disruption alters RGC arbor morphology and increases branch number over days

Our data thus far implicate an autocrine mechanism of Wnt3A signaling that promotes postsynaptic maturation and modulates dendrite morphology. However, Wnts have also been characterized as retrograde factors that facilitate axon guidance in early retinotectal development (Schmitt et al., 2006). Since Wnt3A is expressed in the tectum during retinotectal refinement and various Fz receptors are expressed in the retina, it is plausible that Fz receptors localize to growth cones to mediate transcription-independent effects on axon branches through the divergent Wnt pathways.

Therefore, we sought to determine if Wnt signaling modulates RGC axon dynamics and morphology during retinotopic refinement. To disrupt Wnt signaling in a cell-autonomous manner, we expressed XDsh-ΔPDZ-GFP in RGCs, which has been shown to function as a dominant-negative inhibitor for canonical Wnt signaling in Xenopus embryos (Rothbacher et al., 2000; Sokol, 1996). XDsh-ΔPDZ is a mutant form of the Xenopus Dishevelled-2 protein that lacks the PDZ domain, which is required for signaling in both canonical and noncanonical pathways (Gao & Chen, 2010). Studies have shown the PDZ domain is essential for regulating cytoskeletal rearrangement in axons (Krylova et al., 2000, Ciani et al., 2004, Stamatakou et al., 2015) and dendrites (Rosso et al., 2005, Hiester et al., 2013), therefore we reasoned it would serve as a useful approach for perturbing downstream Wnt signaling cascades. Retinal co-electroporations of XDsh-ΔPDZ-GFP and mCy-RFP were performed to label individual RGC axons that were imaged over 4 days to assess branch growth (**Fig. 3.5A**).

We found that RGC axons expressing XDsh-ΔPDZ showed a significant increase in the numbers of branches that grow over 4 days (**Fig. 3.5C**), and there was a trend towards increased branch length (**Fig. 3.5B**) relative to controls. The density of axon branches per arbor length, on the other hand, was comparable between groups (**Fig. 3.5D**). The increased branch number of XDsh-ΔPDZ axons may be indicative of enhanced exploratory growth of these arbors, suggesting a reduced capacity to stabilize nascent presynaptic contacts—consistent with a role for Wnt signaling in promoting synapse stabilization. Furthermore, XDsh-ΔPDZ axons exhibited irregular morphological characteristics displaying more entangled and tortuous arbors (**Fig. 3.5E**), which could reflect an impairment in directed branch growth within the termination zone. Taken together, these data indicate that dysregulation of Wnt signaling promotes the expansion of RGC arbors, perhaps as a consequence of impaired synapse formation.

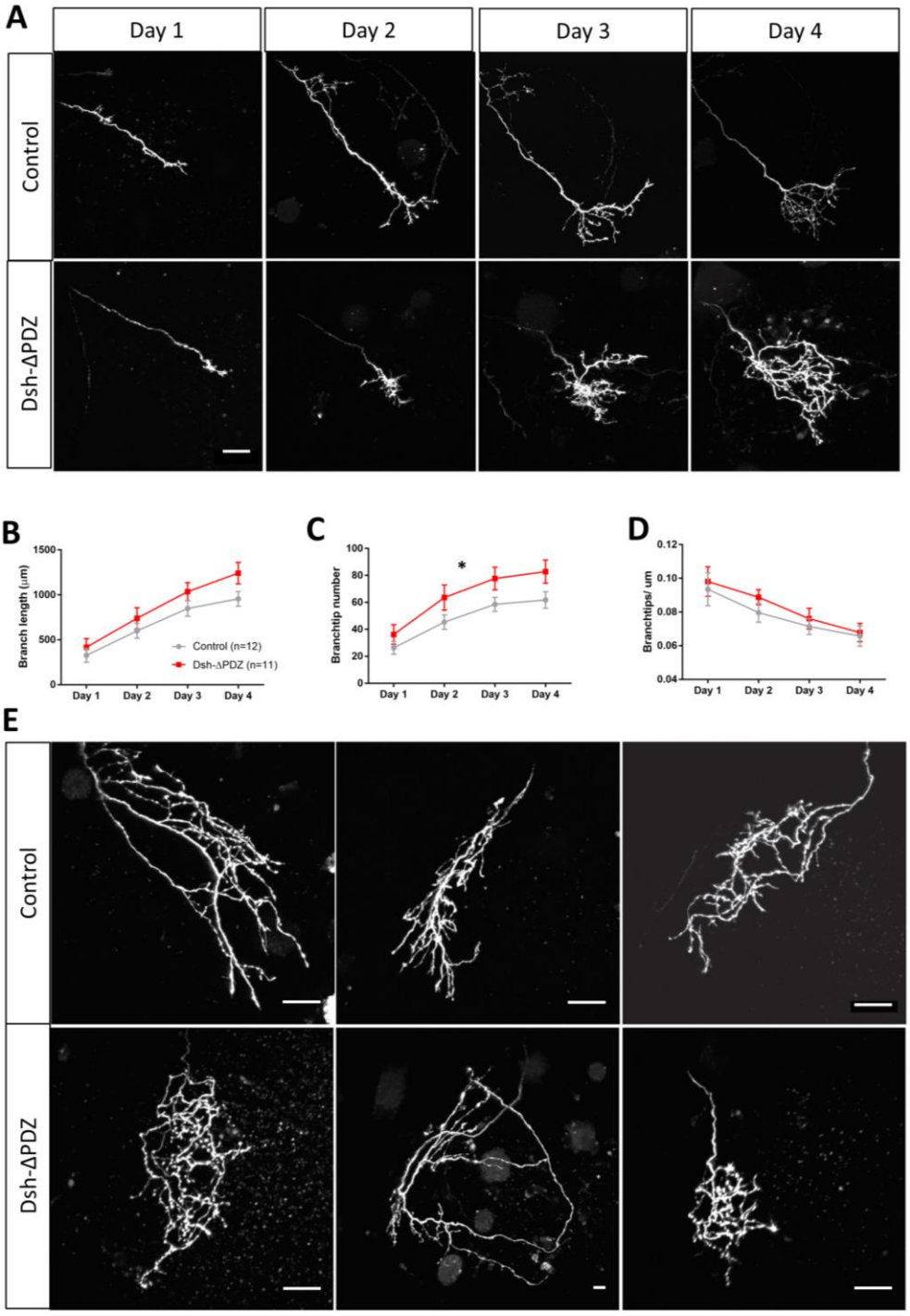

Figure 3.5: Disruption of Wnt signaling in RGCs alters axon arbor morphology. (A) Representative images of control and XDsh-ΔPDZ axons taken over four consecutive days. The scale bar (20μm) applies to all images. (B) Branch length quantification for control (grey) and XDsh-ΔPDZ (red) arbors over four days. (C) XDsh-ΔPDZ axons display a significant increase in branch number (*P=0.047, 2-way ANOVA). (D) Branch density calculated as branch-tips per μm. (E) Examples of XDsh-ΔPDZ and control axons on day 4 of imaging. XDsh-ΔPDZ axons appear more entangled compared to the directional growth exhibited by controls. The scale bar (20μm) applies to all images. Data (B-D) are presented as mean ± SEM.

3.6 Wnt signaling disruption enhances the rate of axon branch growth

There are two possible mechanisms that could account for the observed increase in branch number in XDsh-ΔPDZ-expressing axons: an increase in the addition of branches, or a decrease in the retraction of branches. These competing mechanisms have opposite implications, as the former would suggest that Wnts may normally be functioning as a stabilization cue by suppressing new branch formation, whereas the latter suggests Wnts may normally promote branch destabilization and retraction. To differentiate between these two possible mechanisms, *in vivo* two-photon imaging was performed at a higher temporal resolution to capture the dynamic additions and retractions of axon arbors. Retinal co-electroporations of XDsh-ΔPDZ and mCy-RFP were performed as previously described and images were captured in 10-minute intervals for an hour (**Fig. 3.6A**).

In agreement with the daily imaging experiment, we found that axons expressing XDsh-ΔPDZ displayed a statistical interaction for branch number, growing significantly more branches within 50 minutes of the imaging session compared to controls (**Fig. 3.6B**). Axon length also showed a trend towards increased elongation in axons expressing XDsh-ΔPDZ (**Fig. 3.6C**). However, the number of branch additions (**Fig. 3.6D**) or branch losses (**Fig. 3.6E**) was not significantly different between groups, although there appears to be a greater trend towards decreased branch retractions in XDsh-ΔPDZ axons, with a more subtle trend towards increased branch additions. From these observations, the enhanced growth of XDsh-ΔPDZ axons cannot unambiguously be attributed to alterations in branch additions or losses alone, but likely a combination of these two events. Together, these data affirm that disruption of Wnt signaling in RGC axons enhances the net rate of branch additions and arbor growth, perhaps reflecting an

impairment of Hebbian synaptic stabilization, which we have previously shown to suppress exploratory axonal branching (Munz et al., 2014).

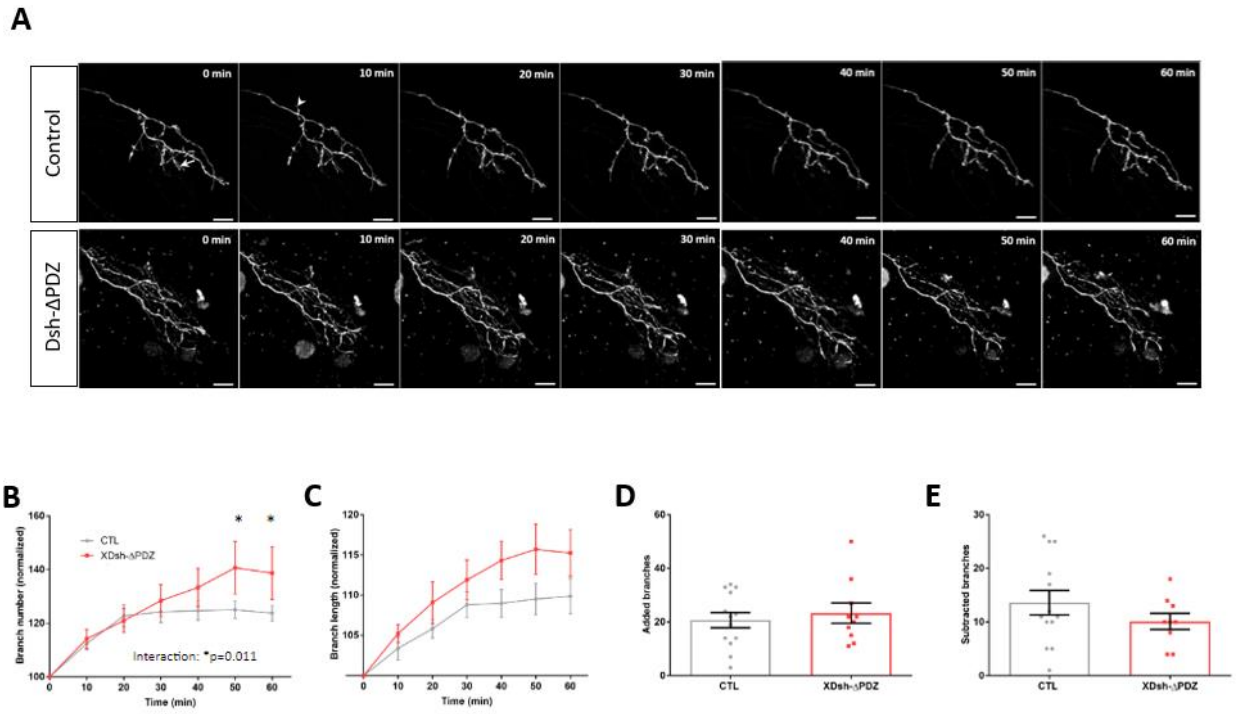

Figure 3.6: XDsh-ΔPDZ axons display enhanced dynamic growth over short time-intervals. (A) Representative micrographs of control and XDsh-ΔPDZ axons showing changes in RGC arbor dynamics over 1 hour. Scale bars are 20µm (B) Comparison of normalized change in axon branch number, showing a significant interaction for XDsh-ΔPDZ axons compared to controls (Interaction: *P=0.011, 2-way ANOVA; multiple comparisons: 50mins: *P=0.023, 60mins: *P=0.030) (C) Comparison of normalized change in axon branch length. (D,E) Total number of branch additions (D) and losses (E) over the course of the 60-minute imaging session. Data are presented as mean ± SEM.

Chapter 4: Discussion

In the retinotectal system of *Xenopus laevis*, visual experience mediates the functional plasticity and morphological remodeling of presynaptic RGCs and postsynaptic tectal neurons alike. Although the postsynaptic mechanisms underlying the detection of patterned activity are better understood, it is unknown how activity-dependent information is transmitted to the presynaptic axon to instruct its growth and stability—suggesting the involvement of one or more activity-dependent retrograde factors. In the present study, we investigated Wnt3A as a candidate retrograde signal for influencing activity-dependent retinotectal remodeling. In retinotectal/retinocollicular circuits, Wnt3/3A is thought to function primarily as a retrograde signal, given its graded expression in the OT/SC and its ability to direct topographic axon guidance via Ryk and Fz receptors on RGC growth cones (Schmitt et al., 2006). Furthermore, overexpression of Wnt3A postsynaptically, but not presynaptically, enhanced region-specific RF plasticity in the OT (Lim et al., 2010). This latter study interpreted this finding as support for Wnt3A as a retrograde factor, however, it is still unclear how Wnts are influencing retinotectal plasticity and whether these effects are presynaptic and/or postsynaptic.

Our data here implicate a novel mechanism and role for Wnt3A in the developing retinotectal circuit as an autocrine signal that facilitates postsynaptic maintenance and maturation. We show that overexpressing Wnt3A in tectal neurons increases mEPSC frequency, AMPA/NMDA ratios, and PSD95 puncta density, suggesting a role for Wnt3A in promoting synapse maturation and/or synapse formation. Furthermore, we observed that Wnt3A overexpression enhances tectal dendrite branch growth, resulting in longer arbors over an 8-hour imaging period. This increase in dendrite growth caused by Wnt3A overexpression can be

attributed, at least in part, to its differential regulation of arbor growth during visual experience, implicating Wnt3A as a modulator of experience-dependent remodeling. Moreover, we used a dominant-negative Dsh construct, XDsh-ΔPDZ, to perturb Wnt signaling in presynaptic RGC axons. We found that disrupting Wnt signaling increased the size of RGC arbors over days and increased the rate of arbor dynamic growth at shorter time intervals. Taken together, these data suggest that Wnt signaling plays an active role at both sides of the synapse during activity-dependent remodeling of the retinotectal circuit. This constitutes a significant advance in our understanding of the multifaceted functions of Wnt signaling that coordinate synaptic connectivity during neural circuit development.

4.1 Wnt signaling regulates RGC axon morphology and structural dynamics

In the retinotectal system, Wnts are considered to act as target derived morphogens that act retrogradely on incoming RGC axons (Sato et al., 2006(b); Schmitt et al., 2006). This is due to the expression of Wnt ligands such as Wnt3/3A in the SC/OT, as well as the expression of Wnt receptors, including Fz and Ryk, in the retina. The graded expression of Wnt ligands and receptors has been found to regulate axon guidance to promote the initial formation of a retinotopic map. In *Xenopus laevis*, Xfz2 receptors are expressed in the retina and mediate axon guidance via sFRP1, a soluble inhibitor of Wnt signaling (Rodriguez et al., 2005). Interestingly, the axonal outgrowth and growth cone turning of RGCs induced by sFRP1 appears to be independent of their antagonist functions on Wnts and are mediated through a distinct process that regulates cAMP and cGMP levels, possibly through noncanonical pathway activation. Despite a clear role for Wnt signaling in axon guidance, it was not understood whether Wnts

influence axon branch dynamics and morphological characteristics during later stages of development. Unlike the topographic mapping phase which relies on chemotropic gradients, the arbor elaboration and refinement phases are thought to be influenced by patterned visual activity which can bias the formation and elimination of nascent synapses along the arbor (Hua & Smith, 2004; Ruthazer et al., 2006). Where these stable synapses are formed determine which axon branches are maintained, therefore the morphological structure and dynamics of axon branches can be treated as a distinct process from topographic termination. Here we provide evidence that Wnt signaling regulates RGC axon dynamics and arbor morphology. Expressing the dominant negative construct XDsh-ΔPDZ promotes the enlargement of RGC arbors over days while increasing their rate of outgrowth during shorter intervals. Given that similar morphological effects are observed when neuronal firing or NMDAR activation is prevented (Munz et al., 2014; Rajan et al., 1999), we interpret the effects of this manipulation to promote the weakening of synaptic connections.

Why would the weakening of synapses cause an expansion of arbors? If anything, one might expect that more stable synapses would increase the number of axon branches retained, and thus result in a larger arbor. A recent study from our lab has shed some light into the mechanisms that govern exploratory axon growth (Rahman et al., 2020). What Rahman and colleagues showed is that neuronal firing generally up-regulates branch loss, resulting in smaller arbors. However, if a given neuron is prevented from firing alongside its neighboring RGC inputs, its rate of new branch additions is increased due to a pro-growth signal secreted by the surrounding RGCs. In other words, axons exhibit exploratory growth (referring to an increase in both branch additions and losses), when their firing is noncorrelated with neighboring RGC inputs. Thus, our observation that XDsh-ΔPDZ promotes exploratory axon growth suggests that

the disruption of Wnt signaling prevents presynaptic inputs from synapsing onto topographically appropriate target neurons, causing them to be firing “out of sync” with their neighboring inputs.

Another mechanistic explanation could involve the influence of homeostatic mechanisms that regulate synapse stability. If synapses are too easily strengthened, this would limit the capacity for inappropriate synapses to be removed. Axonal arbors may prevent this runaway synaptic strengthening through compensatory inhibitory mechanisms that destabilize and weaken adjacent synapses. A study by El-Boustani and collegeaues (2018) showed that heterosynaptic plasticity regulates synaptic strength in the visual cortex of mice. They show that dendritic spines can be selectively potentiated via Hebbian plasticity by pairing a visual stimulus that targets a specific region of the receptive field with the synchronous depolarization of visual cortical neurons using channel-rhodopsin-2 (ChR2). This selective potentiation of dendritic inputs promotes heterosynaptic long-term depression (LTD) at adjacent dendritic spines in a CaMKII-dependent manner, indicating an elegant mechanism whereby synaptic strength is homeostatically controlled to maintain a stable range of synaptic drive. If we presume that a similar mechanism occurs in the retinotectal system, it is plausible that the formation of strong, potentiated synapses will prevent the stabilization of newly formed, weak synapses towards the tips of the axon arbor, thus preventing its outgrowth. If only weak synapses are formed, there will be less of an inhibitory mechanism to weaken adjacent synapses, and thus allow for the arbor to expand and search its territory to find more suitable synaptic partners. Therefore, one could interpret the effects of XDsh-ΔPDZ expression as acting to prevent the stabilization of nascent synapses, resulting in more dynamic and expansive RGC arbors.

How do we know that the morphological effects caused by XDsh-ΔPDZ are not just related to impaired axon outgrowth or guidance? We offer a few reasons why we do not think

this to be the case. The first is that we performed our electroporations of XDsh-ΔPDZ during stages 40-42, after which many nascent RGC projections have arrived at their topographic target zones in the tectum and begin terminal arborization. The added delay of expressing mutant proteins at sufficient levels and trafficking them to presynaptic terminals implies that their effects would occur after stage 42. While this does not entirely preclude a subsequent influence of XDsh-ΔPDZ on axon guidance, Lim and colleagues (2010) showed that inducing the expression of XWnt3A in stage 42 tadpoles did not alter the normal projection zones of ventral or dorsal RGCs in the tectum, suggesting that enhancing Wnt signaling during this period does not influence axon guidance. Another reason we do not believe that our manipulation affects axon guidance is due to complementary findings from a previous group that looked at the effects of different domain deletions of β-catenin (Elul et al., 2003). They found that mutant β-catenin constructs with only the N-terminal domain or lacking the ARM domain (ΔARM) prevented the branching of RGC arbors and resulted in significant mistargeting of these axons in the tectum. On the other hand, a construct harboring only the PDZ domain of β-catenin (at the C-terminal tail) had characteristically large, entangled arbors, although they still targeted the appropriate topographic tectal region. This latter effect is precisely what we see in axons expressing XDsh-ΔPDZ: larger, more entangled arbors. Consequently, we believe that XDsh-ΔPDZ affects axonal arborization rather than topographic targeting, given the delayed activity of our construct after topographic termination, and the finding that mistargeted RGC axons display severely reduced arborization within the tectum—unlike our observations, which show the opposite.

Furthermore, it is unknown whether XDsh-ΔPDZ promotes RGC arbor growth by inhibiting divergent canonical Wnt signaling (referring to the transcription-independent pathways that are mediated by canonical pathways effectors) or by altering other, noncanonical

pathways. The mutant Dsh construct Xdd1, which lacks the same portion of the PDZ domain as our XDsh-ΔPDZ construct, was shown to exhibit dominant-negative inhibition of canonical pathway activation since it abolished the formation of a secondary-axis in *Xenopus laevis* embryos induced by XWnt8 expression (Sokol 1996). This dominant-negative activity for canonical pathway activation appears to be downstream of XWnt8 canonical pathway activation but upstream of β-catenin, as co-injection of mRNA for Xdd1 and β-catenin was still able to promote secondary-axis formation. The expression of Xdd1 by itself disrupts secondary-axis formation, resulting in *Xenopus laevis* embryos having a bent body axis (Rothbacher et al., 2000). The dominant-negative effects of Xdd1 are thought to be due to its ability to form homomeric complexes with endogenous Dsh through their mutual DIX domains, which inhibits the capacity of endogenous Dsh to signal through the canonical pathway via its PDZ domain. However, it was also shown that Xdd1 inhibits the Wnt/PCP pathway by disrupting convergent extension (CE) movements during *Xenopus laevis* gastrulation (Wallingford et al., 2000). Thus, the dominant negative effects of Xdd1/Dsh-ΔPDZ have been shown to impact both canonical and noncanonical PCP signaling in *Xenopus laevis* embryos. However, a recent study has shed some light into the signaling activity of Xdd1, showing that the PDZ domain allows Dsh to adopt an autoinhibitory closed conformation that prevents its activation of noncanonical PCP signaling (Qi et al., 2017). Removal of the PDZ domain allows this mutant XDsh-ΔPDZ to adopt an open conformation that activates PCP signaling. Thus, Xdd1 may not be acting as a loss-of-function, but rather a gain-of-function mutation for PCP signaling. In light of this new study, it is plausible that our XDsh-ΔPDZ construct activates PCP signaling rather than inhibiting it, but it may also prevent canonical pathway activation by preferentially signaling through the PCP pathway, which has been shown to exhibit an autoregulatory inhibition of canonical pathway activation

(Komiya & Habas, 2008). It remains to be determined whether the effects of XDsh-ΔPDZ on RGC axon morphology are due to Wnt/PCP activation or Wnt/β-catenin inhibition (or a combination of the two). Regardless, we believe that the disruption of either of these pathways could account for the axonal morphology phenotypes we observe.

If XDsh-ΔPDZ acts through the inhibition of Wnt/β-catenin signaling and its associated divergent cascades, we posit that it will prevent the Wnt-mediated stabilization of axon branches by increasing cytoskeletal dynamics. For example, Wnt signaling can regulate microtubule (MT) stability and dynamics through Dsh which decreases the phosphorylation of MAP-1B that stabilizes MTs (Krylova et al., 2000, Ciani et al., 2004). By preventing this cascade, MTs would remain dynamic, thereby promoting the expansion of retinal arbors while destabilizing synaptic connections. The activation of Wnt signaling can also induce the translocation of APC to Dsh docking sites at the plasma membrane (Purro et al., 2008). Because XDsh-ΔPDZ lacks the PDZ domain that is essential for its trafficking to the membrane, APC would be retained at the plus ends of MTs to promote the expansion of retinal arbors. Furthermore, the PDZ domain of Dsh is required for its ability to interact with the F-actin binding protein Eps8 (Stamatakou et al., 2015). Preventing this interaction would inhibit the Wnt-mediated increase in F-actin dynamics which could impair synapse formation and maintenance. Thus, the inhibition of divergent canonical cascades via XDsh-ΔPDZ expression is consistent with our morphological observations and the notion that Wnts promote the stabilization and maintenance of synapses.

Another possibility is that XDsh-ΔPDZ acts as a gain-of-function mutant activating Wnt/PCP signaling. The Wnt/PCP pathway has been strongly implicated in postsynaptic differentiation but has also been shown to mediate axon guidance (Onishi et al., 2014). Shafer and colleagues (2011) discovered a novel mechanism for PCP signaling that instructs directional

axon growth in commissural rat explants. They found that Dvl1 antagonizes PCP signaling by binding with Fz3 to prevent its internalization. Upon Fz3-Wnt5a binding, Vangl2 inhibits Dvl1 to promote the internalization of Fz3 where it initiates PCP signaling. This in turn, causes the localized activation of JNK to promote directional, gradient-sensitive outgrowth of commissural growth-cones. Although it remains to be demonstrated whether PCP signaling plays a similar role in the topographic mapping of RGC projections, we posit that the over-activation of PCP signaling could result in increased actin dynamics as well as a loss of the polarized response to Wnt gradients. These effects could account for both the increase in retinal arbor size, as well as the entangled, meandering branches these arbors display.

Thus, whether XDsh-ΔPDZ influences retinal axon morphology by inhibiting canonical Wnt signaling or by enhancing PCP signaling, our observations nonetheless demonstrate that disrupting Wnt signaling in RGCs alters axon branch morphology.

4.2 Wnt3A promotes the functional maturation of retinotectal synapses via a postsynaptic signaling cascade

Due to the characterized roles of Wnts as target-derived factors that instruct retinotopic mapping, it has generally been assumed that Wnts function exclusively as retrograde signaling cues. Our evidence reveals that Wnt3A may also be playing a role at the postsynaptic side of the synapse, implicating it as a bidirectional factor for regulating retinotopic development.

Our electrophysiological evidence shows that Wnt3A overexpression increases mEPSC frequency but not amplitude. The increase in mEPSC frequency suggests that there could either be an increase in the number of functional synapses, or an increased probability of presynaptic release. Our finding that PPR is not altered by Wnt3A expression suggests that this increase in

mEPSC frequency is not due to enhanced release probability. However, our finding that Wnt3A expression increases AMPA/NMDA ratio indicates that Wnt3A could be increasing the number of functional synapses by recruiting AMPARs to postsynaptic sites. And because we do not see an increase in mEPSC amplitude, these observations are best reconciled by a mechanism that involves the enhanced recruitment of AMPARs to NMDAR-only "silent" synapses to promote their functional maturation. In developing neural circuits, a large proportion of synaptic contacts are transiently formed to facilitate a "trial-and-error" process for finding appropriate synaptic connections. These nascent postsynaptic sites often contain NMDARs but lack AMPARs, rendering them silent at hyperpolarized potentials (Cline & Haas, 2008; Kerchner & Nicoll, 2008). The unsilencing of synapses via the recruitment of postsynaptic AMPARs helps to selectively retain correlated inputs that participate in its firing. So just as axonal projections compete amongst themselves for space in the tectum, so too can synapses compete to provide input to a target neuron. We posit that Wnt3A participates in the recruitment of AMPARs—either independently or downstream of neuronal firing—to promote the maturation of nascent synapses. The notion that Wnt3A facilitates postsynaptic maturation is bolstered by the finding that more mature tectal neurons in the rostral tectum display increased mEPSC frequency and AMPA/NMDA ratios than immature neurons in the caudal tectum. Furthermore, the postsynaptic expression of a constitutively active CaMKII—which plays a pivotal role in synaptic potentiation and maturation—mimics the effects of Wnt3A overexpression, showing an increase in mEPSC frequency and AMPA/NMDA ratios (Wu et al., 1996). And given the ability of Wnt signaling to activate CaMKII via the Wnt/Ca^{2+} pathway, it is possible that these two factors mediate their effects through a common signaling cascade.

Consistent with the notion that Wnt3A promotes synaptic maturation, we also find that Wnt3A expression increases the density of PSD95 synaptic puncta in tectal neurons. In the developing retinotectal circuit of zebrafish, tectal dendrites add new PSD95 puncta to filopodia at the tips of actively growing dendritic branches (Niell et al., 2004). While the vast majority of *de novo* PSD95 puncta are promptly retracted, PSD95 puncta that are retained and stabilized serve as anchor points along the actively growing arbor. We propose that an increase in the density of synaptic puncta in Wnt3A expressing tectal dendrites could increase the number of PSD95 puncta that are stabilized and retained instead of being lost. This is further supported by our finding that tectal dendrite length is increased over an 8-hour imaging session in Wnt3A expressing neurons compared to controls. If Wnt3A is promoting the retention of new PSD95 puncta, this would create more anchor points along the arbor from which the dendritic filopodia extend. Since filopodia are often observed to retract back to the point of a stable PSD95 punctum, the generation of stable PSD95 puncta can serve as a positive feedback mechanism for exploratory branch growth (Niell et al., 2004). Indeed, experimental manipulations that disrupt the activity-dependent strengthening of synapses by blocking NMDARs or AMPARs causes a decrease in tectal dendrite arbor size (Haas et al., 2006; Rajan et al., 1999; Sin et al., 2002). Therefore, the increased rate of dendrite arbor growth caused by Wnt3A overexpression could be attributed to their role in synaptic maturation that promotes the retention of new branches.

Another possible explanation for the Wnt3A-mediated increase in synapse density is that Wnts may be promoting synaptogenesis rather than the stabilization of synapses, which are distinct processes (Cline & Haas, 2008). In cultured hippocampal neurons, activity-dependent Wnt release was shown to increase dendritic length through a mechanism involving the

accumulation of β-catenin to sequester N-cadherin (Yu & Malenka, 2003). Since N-cadherin is a transsynaptic adhesion molecule that is involved in early synapse formation and has been shown to induce PSD95 clustering, this presents a plausible pathway by which Wnt signaling could play a role in synaptogenesis (Bamji, 2005; Togashi et al., 2002). Furthermore, similar to what we see with Wnt3A expression, the treatment of tectal dendrites with BDNF was shown to increase the density of synaptic puncta without affecting overall arbor size (Sanchez et al., 2006). This increase in puncta density was caused by an increase in the addition of new PSD95 synapses rather than a stabilization of existing synapses, suggesting a similar mechanism could be at play with Wnt3A overexpression. While further studies are required to discern how Wnt3A is influencing synaptic development, our finding that Wnt3A expression increases mEPSC frequency and AMPA/NMDA ratio is best supported by a mechanism where Wnt enhances the maturation of synapses.

Furthermore, we see that while there is no significant difference in total dendritic branch length between Wnt3A and control neurons under conditions of darkness, there is a significant difference between groups during visual experience, with Wnt3A cells growing longer dendrites compared to controls. There are two explanations for the interaction between Wnt3A expression and visual experience on dendritic branch growth. The first is that Wnt3A could be downregulating the retraction of dendritic branches. Whereas visual activity may normally cause a pruning of inappropriate branches, Wnt3A expression could preferentially stabilize dendritic arbors, thus, negating the impact of visual stimulation on branch retraction. On the other hand, Wnt3A expression could enhance the addition of new branches caused by visual experience. If Wnt3A is released into the synaptic cleft in an activity-dependent manner and promotes dendritogenesis then this could also account for the relative differences in dendritic branch

length. Indeed, this latter mechanism is not without precedent, as Wnt2 has been shown to promote dendritogenesis of rat hippocampal neurons in an activity-dependent manner (Wayman et al., 2006).

While our observations taken in isolation cannot distinguish between these two possibilities, the finding by Lim et al. (2010) that Wnt3A overexpression enhances receptive field plasticity in the optic tectum lends further credence to the notion that Wnt3A potentiates experience-dependent plasticity. Because receptive field plasticity is thought to depend on the selective strengthening of retinal inputs via STDP (Mu & Poo, 2006, Vislay-Meltzer et al., 2006), Wnt3A may acutely facilitate synapse-specific potentiation. What this suggests is that rather than strengthening synapses indiscriminately (which would occur if Wnt3A expression prevents the retraction of inappropriate branches), Wnt3A-mediated plasticity is dependent on correlated activity patterns. Thus, we find it more plausible that visual activity is promoting the release of Wnt3A to enhance the stabilization and elongation of dendritic branches.

If Wnt3A is promoting the functional maturation of postsynaptic sites in an activity-dependent manner, how is it doing so? In the hippocampus, the canonical Wnt ligands Wnt7a/b directly contribute to the recruitment of AMPARs during early LTP expression (McLeod et al., 2018). Induction of LTP promotes the accumulation and release of Wnt7a/b from postsynaptic sites and blockade of Wnt signaling via sFRPs prevents the LTP-induced increase in EPSC amplitude and dendritic spine enlargement, showing Wnt signaling mediates functional and structural plasticity. Wnt7a/b-Fz7 signaling contributes to LTP via the activation of PKA which promotes the phosphorylation of the S845 site on GluA1 subunits to facilitate their trafficking from extra-synaptic sites to the postsynaptic density. Furthermore, Wnt7a/b-Fz7 signaling leads to the activation of CaMKII which causes the loss of SynGAP—a negative regulator of Ras-ERK

signaling. The loss of SynGAP from synapses promotes the localization of AMPARs to postsynaptic sites, illustrating another mechanism by which Wnt signaling mediates early LTP expression. Wnt3A also mediates functional plasticity in the hippocampus, as it was shown to be released in an NMDAR-dependent manner to facilitate LTP (Chen et al., 2006). The mechanisms underlying the activity-dependent release of Wnts are still unclear, however one likely mechanism is through the release of exosomes, as exosomes have been shown to be released in an activity-dependent manner and harbor active Wnt ligands (Budnik et al., 2016). Putting this together, we propose a model for Wnt signaling whereby Hebbian plasticity through NMDAR-activation promotes the release of Wnt3A from postsynaptic sites which, in turn, facilitates the recruitment of AMPARs to nascent synapses—possibly via the activation of CaMKII.

4.3 Implications of region-specific Wnt signaling on circuit remodeling

Our data support a role for Wnt3A in facilitating retinotopic refinement, however, one obvious question remains to be addressed: if Wnts are expressed as a gradient in the OT, how can they function as global activity-dependent retrograde cues? Indeed, the graded pattern of Wnt expression in the OT (Wolda et al., 1993, Lim et al., 2010) and our observations of a graded activation of the canonical Wnt pathway suggest that Wnts are acting in a region-specific manner. Although this very well may be the case, we should also note that the expression patterns of the full complement of Wnt ligands—of which there are 17 in *Xenopus laevis* (https://web.stanford.edu/group/nusselab/cgi-bin/wnt/xenopus)—and their receptors have not been characterized in the retina or tectum during retinotectal development. This means we cannot rule out the function of other Wnt ligands beyond Wnt3A, some of which could be expressed

more homogenously across the tectum. Indeed, a study looking at the tissue-specific expression of RNA transcripts in *Xenopus laevis* reveals a large array of transcripts for Wnt ligands and receptors in the eye and brain of mature animals, although the precise regions in the brain and eye where these transcripts are localized remains unknown (Michiue et al., 2017). Another reason to believe that Wnt signaling may have a more global function during retinotectal development is the expression pattern of Fzs in RGCs: unlike Ryk receptors—which contribute to topographic mapping and are expressed in a gradient—Fz3 is expressed homogenously in the RGC-layer, which could indicate they serve a broader purpose during circuit development (Schmitt et al., 2006). Whereas high concentrations of Wnt3 activate Ryk receptors to promote chemorepulsion in mammalian retinal explants, Fz3 appear to be more sensitive to low concentrations of Wnt3 to facilitate outgrowth. In *Xenopus laevis* embryos, Xfz2 is localized exclusively to the RGC layer and displays a homogenous expression pattern (Rodriguez et al., 2005). Xfz5 also displays eye-specific expression in *Xenopus laevis* embryos (Sumanas & Ekker, 2001) and given the finding that Fz5 undergoes activity-dependent localization at presynaptic terminals in the hippocampus to promote presynaptic differentiation (Sahores et al., 2010), it is plausible that Xfz5 could be playing a similar role during retinotopic development. Thus, the uniform expression of Fz homologs in the retina of *Xenopus laevis* tadpoles indicate that they may contribute to additional functional roles during retinotopic development beyond topographic mapping.

An even more tantalizing possibility is that complementary gradients of Wnt3A and EphB in the OT (Higenell et al., 2011) mediate their effects through a common pathway (Cheyette, 2004). Dsh not only transduces Wnt signaling events but has also been shown to associate with EphB and ephrin-B to mediate both forward and reverse signaling (Tanaka et al.,

2003). Expression of the dominant-negative Xdd1 in *Xenopus laevis* embryos disrupts endogenous EphB/ephrin-B signaling by preventing the activation of RhoA. And because RhoA is a well characterized effector of the Wnt/PCP pathway, the activation of Xdsh and RhoA may serve as a common biochemical cascade for transducing Wnt and ephrin signaling. Thus, what appear to be distinct Wnt3/EphB gradients in the OT may actually depict a homogenous set of ligands that converge to co-activate a common presynaptic pathway. While the graded expression of these molecules may still guide topographic mapping via a separate signaling cascade, they may also serve an auxiliary function of coordinating presynaptic differentiation and/or plasticity. Although still speculative, the demonstration of convergent retrograde signaling via distinct ligands, Wnt3 and EphB, would highlight a role for signaling pathway crosstalk as a mechanism for neural circuit development.

Finally, we must also consider the possibility that Wnts function in a region-specific manner. It is generally assumed that given the broad requirement for an activity-dependent retrograde signal, such a mechanism will operate uniformly throughout the tectum. However, it remains possible that there are many distinct molecular players and mechanisms that orchestrate the activity-dependent refinement of presynaptic terminals. Wnts, along with other retrograde factors—both characterized and uncharacterized—may act in concert to coordinate the various molecular events that precipitate presynaptic maturation. Thus, there may not be one molecular factor that is both necessary and sufficient for activity-dependent retrograde signaling in the OT, rather, there are a complement of factors that are regulated across space and time, each playing their own role.

4.4 Wnts as multifunctional tools for instructing topographic refinement

The process of biological development is one of extraordinary dynamism and plasticity. This dynamism can be most readily observed by the morphogenesis of organisms' tissues and body-plans however, we may suspect that the underlying molecular signaling networks are similarly dynamic. As mentioned in the first chapter, the ubiquity and diversity of Wnt signaling components render them uniquely suitable for fulfilling versatile cellular roles. Our evidence here supports the notion that Wnt3A has various functional roles during retinotectal development, contributing to topographic mapping as well as arbor dynamics and synaptic maturation. What does this system achieve by involving Wnts in so many distinct processes? One explanation could be energetic efficiency. The transcription and translation of Wnts and their associated signaling components is a metabolically costly process, and so it is plausible that these vast signaling networks can be repurposed to dynamically regulate events that are both transient and persistent. Retinotopic map formation is one such example of a developmental process that proceeds through sequential—but partially overlapping—stages that are transient, first starting with topographic termination, followed by arbor elaboration and structural/functional refinement. Thus, the participation of Wnts throughout these various phases constitutes an elegant mechanism for maximizing their functional utility in an energy-efficient manner.

The functions of Wnt signaling are not only regulated across time, but space as well. Our evidence suggests that Wnts play distinct functional roles at both pre and postsynaptic sites. Whereas Wnt signaling influences axonal targeting by regulating their structural dynamics, Wnts appear to play a more prominent role in promoting synaptic maturation in postsynaptic dendrites. This is the first characterization, to our knowledge, of bidirectional functions of Wnt signaling

during retinotectal development. However, the capacity for Wnts to initiate signaling cascades at both sides of the synapse has been well documented in other systems like the drosophila NMJ and rodent hippocampus (Packard et al., 2002; Teo & Salinas, 2021).

Thus, our data illustrate the diverse functional capabilities endowed by Wnt signaling in developing systems. It is the dynamic regulation of Wnt signaling networks and their biochemical cascades that are likely responsible for its pleiotropic effects, demonstrating the principle that one molecular factor can have many distinct roles.

4.5 Caveats and Limitations

There are a few additional caveats in these experiments that will be mentioned here. Our retinal electroporations of the XDsh-ΔPDZ construct were performed with the intention of disrupting Wnt signaling in RGC axons however, it is possible that the expression of this construct could also interfere with Wnt signaling in RGC dendrites and soma which could partially account for the morphological differences we observe. Although this is a possibility, our finding that canonical Wnt signaling activity appears to be absent from the RGC layer suggests that Wnt signaling may not be as prevalent in the RGC soma compared to RGC axons. We also made sure to confirm the presence of GFP-tagged XDsh-ΔPDZ in retinal axons to ensure their proper expression and targeting to axon arbors. Another possibility is that our retinal electroporations of XDsh-ΔPDZ resulted in the expression of this construct in many non-RGC cell-types, which could have disrupted visual processing and activity in the retina. It is likely that the electroporations themselves had more of a disruptive impact on visual processing than the construct expressed, thus we presume that both control and XDsh-ΔPDZ-expressing animals are similarly affected.

One implication of Wnt3A being expressed as a gradient in the tectum is that the effects of the expression of our dominant-negative XDsh-ΔPDZ may be more pronounced in ventral RGCs which project to the dorsal tectum, where Wnt levels are high, whereas these effects will be muted in more dorsal RGCs that project to regions where Wnt levels are low. The procedure of retinal electroporations gives little control over the location of RGCs in the retina that express our desired construct, and thus we expect that our group data will comprise a random distribution of RGCs along the D-V axis of the retina. If we presume that XDsh-ΔPDZ functions by impeding the transduction of Wnt3A signaling, then it follows that our group data may underestimate the impact of XDsh-ΔPDZ on dorsally projecting RGCs. In other words, we anticipate that the effects of XDsh-ΔPDZ will be region-specific and dependent on the Wnt3A concentrations within their respective termination zone.

As a more general limitation, it should be acknowledged that although we see distinct effects of Wnt signaling on the pre and postsynaptic side, this does not rule out the possibility of Wnt3A having a purely pre or postsynaptic locus of action. It is possible that the effects we observe of Wnt signaling on postsynaptic maturation could be mediated by a recruitment of presynaptic adhesion molecules that facilitate the subsequent maturation of postsynaptic contacts. On the other hand, the effects of Wnt on RGC arbor growth could be mediated by a postsynaptic mechanism where Wnt activity is promoting the secretion of a downstream retrograde signal or recruiting transsynaptic adhesion molecules that interact with presynaptic terminals. As such, it is extremely difficult to fully dissect the sequence of events by which Wnt signaling activation promotes synapse maturation and where these events first occur. Nonetheless, we still find it highly likely Wnt signaling plays an active role at both sides of the synapse given that our presynaptic manipulations were cell-autonomous—therefore having no

direct effect on the postsynaptic secretion of Wnt3A—and we observed canonical Wnt signaling activity in postsynaptic tectal neurons, implying these cells have the capacity to transduce Wnt signaling events.

4.6 Summary

In the present study, we demonstrate that Wnt3A promotes the functional maturation of synapses in the developing retinotectal circuit. We show that Wnt3A overexpression increases mEPSC amplitude, AMPA/NMDA ratios, and the density of PSD95 synaptic puncta, suggesting Wnt3A enhances postsynaptic maturation, likely through the recruitment of AMPARs to nascent synapses. We also show that Wnt3A enhances the growth rate of tectal dendrites in an experience-dependent manner, consistent with the notion that Wnt3A is stabilizing nascent postsynaptic contacts.

Furthermore, we show that the disruption of presynaptic Wnt signaling via the expression of a dominant-negative XDsh-ΔPDZ promotes an enlargement of RGC axon arbors in a cell-autonomous manner. The enlarged arbors displayed by XDsh-ΔPDZ translates to an increased rate of dynamic growth, that likely reflects the impairment of retrograde Wnt signaling to promote the stabilization of axon arbors.

Given these findings, we propose the following model whereby Wnt signaling promotes synapse stabilization and maturation through separate presynaptic and postsynaptic pathways. Upon the activation of postsynaptic NMDARs Wnt3A is released into the synaptic cleft where it acts on Wnt receptors, likely Fzs, on both sides of the synapse. On the postsynaptic side, Wnt3A recruits AMPARs via a mechanism that could involve the downstream activation of CaMKII.

which causes the loss of SynGAP from synaptic sites and the subsequent localization of AMPARs (McLeod et al., 2018). Alternatively, Wnt3A may also activate PKA to phosphorylate the S845 site on GluA1 subunits to facilitate their trafficking from extra-synaptic sites to the postsynaptic density. This would facilitate the stabilization and maturation of synapses to increase the density of postsynaptic sites and the growth rate of tectal dendrites.

On the presynaptic side, Wnt3A travels across the synapse to activate Fz receptors in RGC axon terminals. This promotes the stabilization of axon microtubules via the subcellular localization of APC to the plasma membrane (Purro et al., 2008). This would stabilize axon branches to facilitate the formation of presynaptic contacts by recruiting transsynaptic adhesion molecules like N-cadherin (Bamji, 2005).

Although this mechanistic model is speculative, it provides a plausible interpretation of our data that clearly demonstrate a role for Wnt signaling at both sides of the synapse, participating in distinct functional roles. Nonetheless, there are still many questions that remain to be answered. How does activity regulate Wnt signaling in the retinotectal circuit? Does postsynaptic depolarization trigger the release of Wnts into the synaptic cleft, or does it regulate the subcellular localization of Wnt signaling components at the synapse? To what extent do Wnt transcriptional pathways influence the maturation of synapses? And to what extent is the transcription of Wnt signaling components regulated over the course of development? Are Wnts synaptogenic, or do they only promote the maturation of existing synapses? The complexity of Wnt signaling ensures there are no easy answers to these questions, and yet answering these questions has significant implications for uncovering how Wnts coordinate synaptic development in the retinotectal system and beyond.

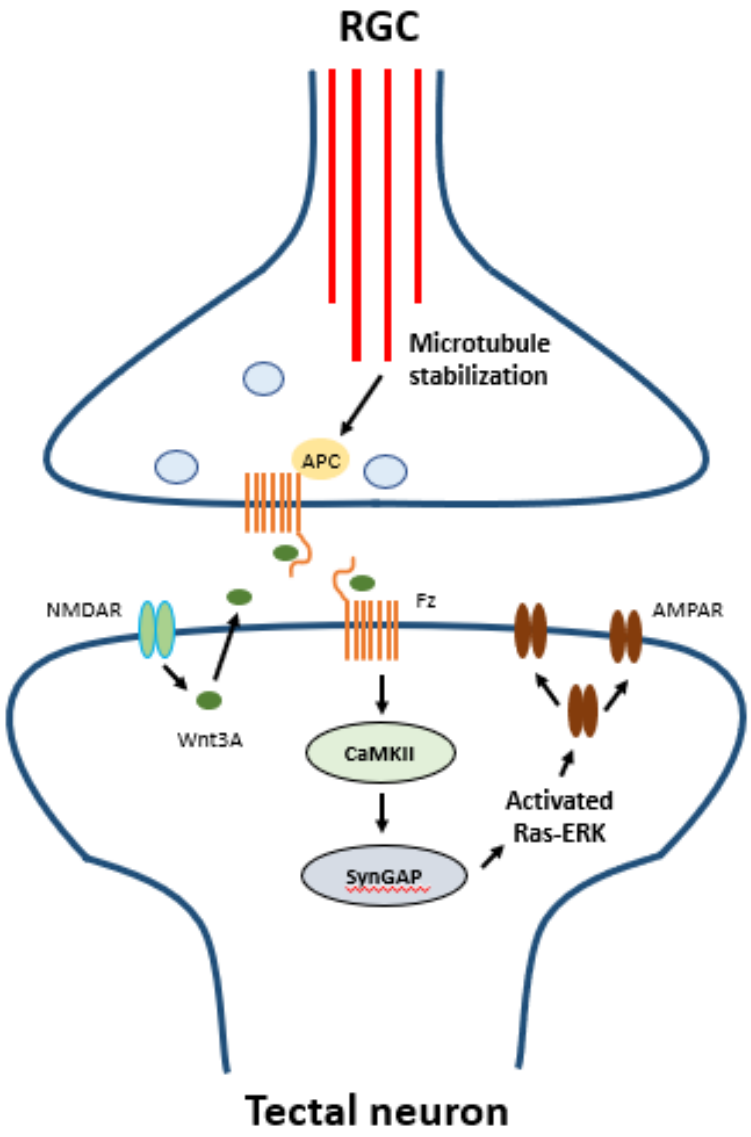

Figure 4.1: Proposed model for Wnt signaling during retinotectal remodeling. Wnt3A is released in an activity-dependent manner from postsynaptic tectal neurons downstream of NMDAR activation. On the postsynaptic side, Wnt-Fz signaling could promote the activation of CaMKII and downstream Ras-ERK signaling to facilitate the recruitment of AMPARs to nascent synapses. On the presynaptic side, Wnt-Fz signaling can cause the translocation of APC from microtubule tips which would stabilize both microtubules and presynaptic terminals. The design of this figure was inspired by (Teo & Salinas, 2021).

Contributions to Original Knowledge

To the best of our knowledge, our data here provide the first demonstration of bidirectional Wnt signaling during retinotopic development. Wnts have hitherto been recognized exclusively as retrograde factors, however our data propose a more complex model for the function of Wnts in retinotectal remodeling. The utility of Wnts go beyond their mere roles in early topographic termination, allowing them to orchestrate developmental processes at both sides of the synapse.

We also show a distinct role for Wnt signaling in regulating axonal morphology, which is thought to be mechanistically distinct from its role in guiding topographic termination. Thus, not only can Wnts provide positional information to instruct topographic mapping but can also regulate terminal axon branching within these termination zones.

These data have broad implications on the diverse functional roles of Wnts in the developing nervous system that can be dynamically regulated across space and time. We propose that the multifunctional roles of Wnts depict an elegant strategy that conserves metabolic energy by repurposing Wnt signaling networks to mediate distinct cellular events. In the context of developing neural circuits, we suspect that Wnts may have diverse roles in other systems as well given their functional utility, diversity and dynamic regulation.

Finally, these data have important implications for the multifaceted roles of Wnt signaling in coordinating synaptic connectivity in developing neural circuits. Given that neurodevelopmental disorders have been attributed to aberrant synaptic connectivity and many Wnt genes are associated with these developmental disorders, our data shed further light on the essential role Wnts play in establishing synaptic connectivity and may yield targets to help treat or prevent these neurodevelopmental disorders.

Concluding Remarks

I've always had an interest in understanding how things work at a fundamental level. This project is an extension of that curiosity, applied to understanding how it is that experiences can shape the structure and function of developing nervous systems. In the service of this goal, I have subscribed to the idea that complex systems like the brain can be understood by reducing them to their most fundamental constituents—cells and molecules—and characterizing how these components interact.

In the years of doing this project, I've had to grapple with and confront the limitations of this reductionist approach. When diving down the rabbit hole of Wnt signaling, one quickly finds that the neat schemas for molecular signaling pathways you see in textbooks are woefully inadequate to capture the complex reality that takes place in a living organism. Molecular pathways—like biological organisms—cannot be treated as standalone, independent entities, but are heavily influenced by the context and environmental milieux in which they arise. And thus, no understanding of developmental mechanisms is complete without characterizing the full complement of dynamic interactions between genes, molecules, signaling pathways, cells, and environment, which will always be limited to where and how we look.

This is not to diminish the remarkable progress that has been made in understanding how these components come together during the process of development—and this is no small feat. I believe we can appreciate how far we've come in mapping the mechanisms of biological development, while at the same time acknowledging that the map is not the territory. As the aphorism goes, 'All models are wrong, but some are useful'. It is my hope that this work may indeed be useful.

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