CADHERIN INVOLVEMENT IN AXONAL BRANCH STABILITY IN THE *XENOPUS* RETINOTECTAL SYSTEM

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I dedicate this thesis to my family.

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ABBREVIATIONS

CBR – catenin binding region

CFP – cyan fluorescent protein

Eph – erythropoietin-producing hepatocellular receptor

(e)GFP – (enhanced) green fluorescent protein

(e)YFP – (enhanced) yellow fluorescent protein

mCherry – a monomeric red fluorescent protein

N-cadΔE – dominant-negative N-cadherin lacking extracellular domains (1 to 5)

NMDA – N-methyl D-aspartate

PDZ – postsynaptic density disk zona occludens-1

RGC(s) – retinal ganglion cell(s)

RNAi – Ribonucleic Acid (RNA) interference

Syp – synaptophysin

TTX – tetrodotoxin

XNcad-GFP – *Xenopus* N-cadherin fused to eGFP at C-terminus

ABSTRACT

Retinal ganglion cell (RGC) axon arbors within the optic tectum are refined in development through a dynamic process of activity-dependent remodeling. The synaptic adhesion molecule N-cadherin is a candidate for mediating selective stabilization and elaboration of RGC axons due to its localization to perisynaptic sites and its modifiability by neural activity. RGCs of *Xenopus* tadpoles were co-transfected with plasmids encoding a dominant negative N-cadherin (N-cadΔE) and eGFP or eYFP. Using two-photon *in vivo* time-lapse imaging, we found that axons expressing N-cadΔE became less elaborate than controls over three days of daily live imaging. Shorter interval time-lapse imaging of axons expressing synaptophysin-GFP to visualize putative synaptic sites revealed that N-cadΔE expressing axons form fewer stable branches than controls and that stabilization of axonal branches at synaptic sites is altered. We conclude that N-cadherin participates in the stabilization of axonal branches in the *Xenopus* retinotectal system.

RESUMÉ

L'arborescence des cellules ganglionnaires de la rétine (CGR), qui projètent leurs axones dans le tectum optique, se raffinent par un système dynamique de remodelage dépendant de l'activité visuelle. La N-cadhérine, une protéine d'adhésion synaptique, pourrait orchestrer l'élaboration et la stabilisation sélective des projections axonales des CGRs. En effet, sa localisation dans la région périsynaptique et son changement de conformation en fonction de l'activité neuronale en font un candidat particulièrement attrayant. Afin de tester cette hypothèse, nous avons co-transfecté des CGRs de têtards *Xenopus* avec des plasmides exprimant un dominant négatif de N-cadhérine (N-cadΔE) et du eGFP ou eYFP. À l'aide d'un microscope biphotonique prenant des images à intervalles fixes, nous avons suivi l'évolution des CGRs sur une période de trois jours, et découvert que les axones exprimant la N-cadΔE sont moins élaborés que ceux exprimant seulement eGFP/eYFP. Pour visualiser les sites synaptiques putatifs, nous avons exprimé la Synaptophysin-GFP et utilisé une imagerie à intervalles plus courts. Les axones exprimant N-cadΔE forment moins de branches stables que les contrôles, et la stabilisation de leurs branches aux sites synaptiques est également diminuée. Nous en concluons que la N-cadhérine participe à la stabilisation des branches axonales du système rétinotectique du *Xenopus*.

CHAPTER ONE.

Background & Project aims

In the development of the information highways of our central nervous system, forming the circuitry involves a variety of neural sprouting, connectivity, and branching - but also a significant amount of pruning and reorganization. This is important in the development, maintenance, and constant rearrangements in the circuitry of our central nervous system. (Katz & Shatz, 1996)

• Retinotectal system development

The connectivity of our neural system can be investigated and better understood using model systems that permit systematic manipulation of putative mechanisms. The retinotectal system, an evolutionarily primitive vertebrate brain circuit involved in vision, is an ideal candidate to investigate the projections of axons within brain matter because of our ability to manipulate both presynaptic and postsynaptic environments separately. Namely, the axonal projection of retinal ganglion cell (RGCs) onto the optic tectum permits us to label and manipulate axon terminals within visual brain areas.

Visual input from photoreceptors, through bipolar and amacrine cells are integrated within the RGC layer. In development RGC axons are guided by a series of molecular guidance cues to reach their termination zone in the optic tectum successfully. This is first observed at the level of the retina, as RGCs enter the optic nerve head a combination of laminin and netrin-1 creates an environment that 'attracts and pushes' the converging axons into the netrin-1 rich optic nerve head. Netrin-1 is further observed along the visual pathway as a chemo-deterrent aiding growth cone guidance once RGC axons exit the eye (for review see Mann, Harris, & Holt, 2004). An explanation for this change in affinity for netrin-1 is believed to take place in the RGC growth cones

themselves; as a change in the specific developmental stage of growth is linked to a response to environmental molecular cues (Shewan, Dwivedy, Anderson, & Holt, 2002). Diagram I (adapted from Lemke & Reber, 2005) illustrates the path of retinal ganglion cells from the retina, through the optic nerve head, across the optic chiasm and to their respective tectal termination zones – depending on their retinal location.

The ephrin family of ligands and Eph receptors provide a signaling mechanism that successfully links retinal concentration gradients of RGCs onto complementary tectal cell counterparts. This is effectuated by a ligand/receptor cell-cell interaction between RGC axon terminals and the tectum. Ephrin-A's bind to Eph-A receptors to mediate a repulsive interaction, while Ephrin-B's bind to Eph-B receptors to form an attractive interaction. Retinotopic mapping results from the EphrinB/EphB gradient running dorsal to ventral, while the EphrinA/Eph-A gradient run rostral to the caudal (or nasal to temporal) as seen Diagram I B. Differences in substrate and receptor concentration in pre- and post-synaptic partners enable a Cartesian-like coordinate pattern enabling specific retinal inputs to terminate accordingly within complementary tectal tissue (please see Lemke & Reber, 2005; Mann et al., 2004 for review). After this crude map formation, a further pruning of the axonal branch to a precise and distinct zone appears to occur.

The activity-dependent remodeling and fine-tuning of the projections is clear in several studies involving the disruption of retinal activity leading to a disruption of map formation. A gradual shifting of the termination sites of RGC axons across the developing visual map, requiring an ongoing change in pre- and postsynaptic binding partners, is a characteristic of the developing fish and amphibian brain (Easter, Rusoff, & Kish, 1981; Raymond, Easter, Burnham, & Powers, 1983; Reh & Constantine-Paton, 1985). This is a consequence of the fact that the eye and brain both grow differentially

throughout life adding new cells radially at the proliferative lip of the retina and linearly along the ventricular zone of the optic tectum (Gaze, Keating, & Chung, 1974). Because RGC axons must continually make and break connections as their projections shift across the tectal neuropil, activity-dependent refinement of axon terminals continues throughout life and is utilized by the regenerating projection following optic nerve crush as well. In goldfish, blockade of retinal spiking activity by TTX eye injections, prevents the formation of discrete patches of regenerating RGC terminal inputs onto the tectum (Olson & Meyer, 1991). Furthermore, disruption of synchronized activity by strobe rearing during regeneration leads to a decrease in RGC arbor refinement (Schmidt & Buzzard, 1993). While most initial studies were carried out in regenerating retinotectal projection, the requirement of neural activity for retinotectal map refinement in normal development has also been demonstrated by pharmacological blockade of neural activity in frogs (Reh & Constantine-Paton, 1985), chicks (Kobayashi, Nakamura, & Yasuda, 1990), and rodents (Simon, Prusky, O'Leary, & Constantine-Paton, 1992); leading to our understanding that across vertebrates activity dependent map refinement occurs. Genetic mutants having altered neuronal activity have provided further compelling evidence for activity playing a key role in retinotectal map refinement in zebrafish (Gnuegge, Schmid, & Neuhauss, 2001) and mouse (McLaughlin, Torborg, Feller, & O'Leary, 2003).

However, the fact that patterned neural activity is instructive (i.e., carrying the very information used by the system to alter its connectivity in the patterns of action potential firing) in map refinement first became clear in experiments in which a third eye was implanted in a *Rana pipiens* frog larva. The extra eye successfully projected onto an otherwise mono-innervated tectum, creating segregated patches of inputs for each eye in that tectum. Because blockade of retinal activity by the application of a TTX cuff on the

optic nerve resulted in desegregation of the inputs, it was concluded that their ability to segregate was a consequence of afferents from the two eyes carrying different activity patterns. This reinforces the idea of an instructive role of RGC axons in the development of the retinotectal map as there is no reason the normally monocular innervated tectum would carry molecular cues for the segregation of visual inputs (Reh & Constantine-Paton, 1985).

Retinal arbor morphology and function is not only dependent upon firing by the presynaptic RGC axons but also involves postsynaptic tectal cell activation through N-methyl-D-aspartate receptor (NMDAr) activity. Synchronous activity of multiple inputs relieves a Mg+ block of NMDArs to allow calcium permeability. Blockade of NMDArs causes a disruption of map formation confirming to the idea that it may be a key player in activity-dependent map refinement and demonstrating that retrograde signaling from post- to presynaptic cells must occur during map refinement (Cline & Constantine-Paton, 1989; E. S. Ruthazer, Akerman, & Cline, 2003; Schmidt, Buzzard, Borress, & Dhillon, 2000).

RGC axonal arbors consist simultaneously of stable, extending, and retracting branches. However, all these dynamic branches have points of stability in their quest to optimize connectivity. Mature synapses appear to serve as anchoring points in the dynamic remodeling of these branches as revealed by the finding that although branches can readily retract past immature synapses, they are relatively stabilized at mature ones. Thus, the formation of stable synapses regulates the dynamic behavior of axonal branches and may be essential for the activity-mediated fine-tuning of the retinotectal mapping (Meyer & Smith, 2006; E. S. Ruthazer, Li, & Cline, 2006). However, little is known about how synaptic contacts might stabilize axonal arbor structure.

• N-cadherin is a candidate for mediating arbor stabilization

The synaptic molecule N-cadherin is a good candidate for mediating axonal arbor stabilization in development. N-cadherin is a cell adhesion molecule containing five extracellular cadherin repeats, the most distal one (EC-1 region, paticuarlyTrp2) is important in binding specificity (Boggon et al., 2002). The molecule has a single transmembrane domain and an intracellular catenin-binding region thought to link the intracellular cytoskeleton via catenin proteins to the extracellular environment. N-cadherin is expressed in retinal ganglion cells and found to be important for axonal initiation, outgrowth (Riehl et al., 1996), and guidance (Matsunaga, Hatta, Nagafuchi, & Takeichi, 1988). Within the tectal neuropil, N-cadherin takes a punctate appearance and colocalizes with synaptic markers in RGCs (Uchida, Honjo, Johnson, Wheelock, & Takeichi, 1996). There is some evidence cadherins may be important in synapse stabilization and maturation. Block of N-cadherin function by expression of a dominant negative results in delayed synaptogenesis and synapse maturation (Huntley, 2002; Latefi, 2006). This in turn affects the kinetics and physiology of the synapse (Jungling et al., 2006) . Hippocampal slice work revealed a decrease in excitatory postsynaptic potentials and the prevention of long-term potentiation induction in the presence of function blocking N-cadherin antibodies (Bozdagi, Shan, Tanaka, Benson, & Huntley, 2000; Tang, Hung, & Schuman, 1998). The location of N-cadherin at the perisynaptic junction and its role in the aforementioned events leads us to consider the ability of this molecule to change its confirmation with synaptic activity to enhance the connection of the binding partners it interacts with.

Cadherin is trafficked to new putative synaptic sites in developing axons (Jontes, Emond, & Smith, 2004) where it can be found in a complex with catenins and other

proteins (Ozawa, Baribault, & Kemler, 1989). In one influential model, N-cadherin is thought to interact via β-catenin to αN-catenin, which is known to interact with F-actin and α-actinin (Knudsen, Soler, Johnson, & Wheelock, 1995; Rimm, Koslov, Kebriaei, Cianci, & Morrow, 1995). Upon phosphorylation by cyclin dependent kinase-5, β-catenin itself dissociates from the catenin-cadherin complex (Schuman & Murase, 2003). In hippocampal culture, regulation of the phosphorylation state of β-catenin effects an activity-dependent modulation of the formation of the cadherin/catenin-signaling complex. Upon membrane depolarization and NMDAr activation, β-catenin is dephosphorylated and translocates to cadherin rich synaptic sites of dendritic spines (Murase, Mosser, & Schuman, 2002; Schuman & Murase, 2003). In this way, postsynaptic changes in N-cadherin signaling can be modulated by presynaptic neural activity.

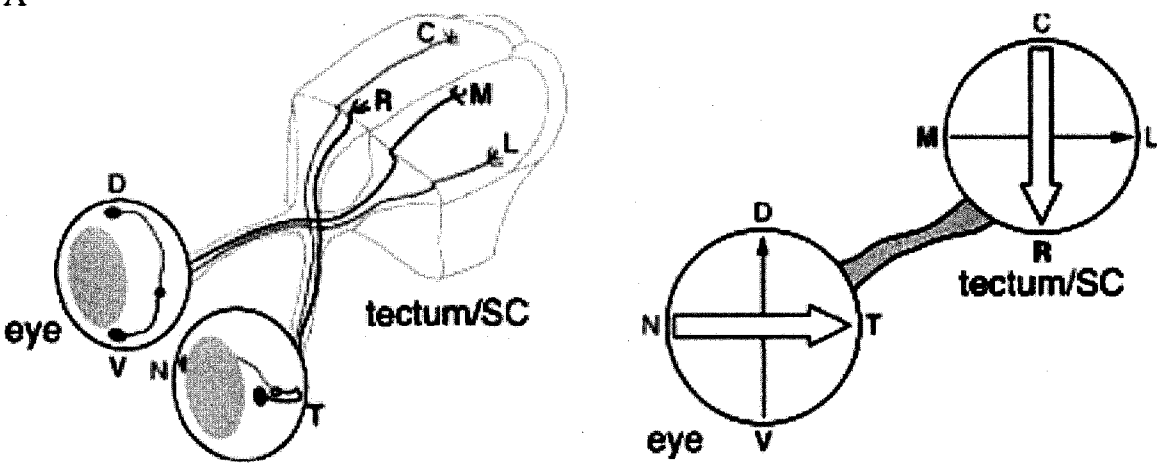
Furthermore, it has been shown that rapid dimerization of N-cadherin occurs by NMDAr activation and neuronal depolarization (Tanaka et al., 2000). This assembly into dimers is important in order to create adhesive *cis*-dimers from N-cadherin monomers. NMDAr activation also results in increased protease resistance of N-cadherin, potentially enabling its longer presence at synaptic sites. It is important to note that the α/β-catenin complex is known to aggregate to synaptic sites upon NMDAr activation, and is thought to play a role in the dimerization of N-cadherin (Imamura, Itoh, Maeno, Tsukita, & Nagafuchi, 1999; Murase et al., 2002). A graphical representation of the development of CNS synapses is shown in Diagram II. In panel A, diffuse N-cadhrerin/β-catenin complexes are found in the pre/post synaptic zones as well as a translocation towards the active zone of synaptic vesicles and piccolo bassoon transport vesicles (PTV), containing synaptic proteins including N-cadherin. Cell-to-cell contact, as shown in panel B, links

pre- and post- synaptic partners to their respective actin cytoskeletons. Finally, a mature synapse in panel C suggests the recruitment of PDZ domain containing proteins, glutamate channel recruitment, and the perisynaptic localization of N-cadherin around the active zone (Bamji, 2005).

The maturation of synapses thus could be linked to an activity-dependent dimerization of N-cadherin, involving a number of key players that would link the actin cytoskeleton to extracellular adhesion through N-cadherin.

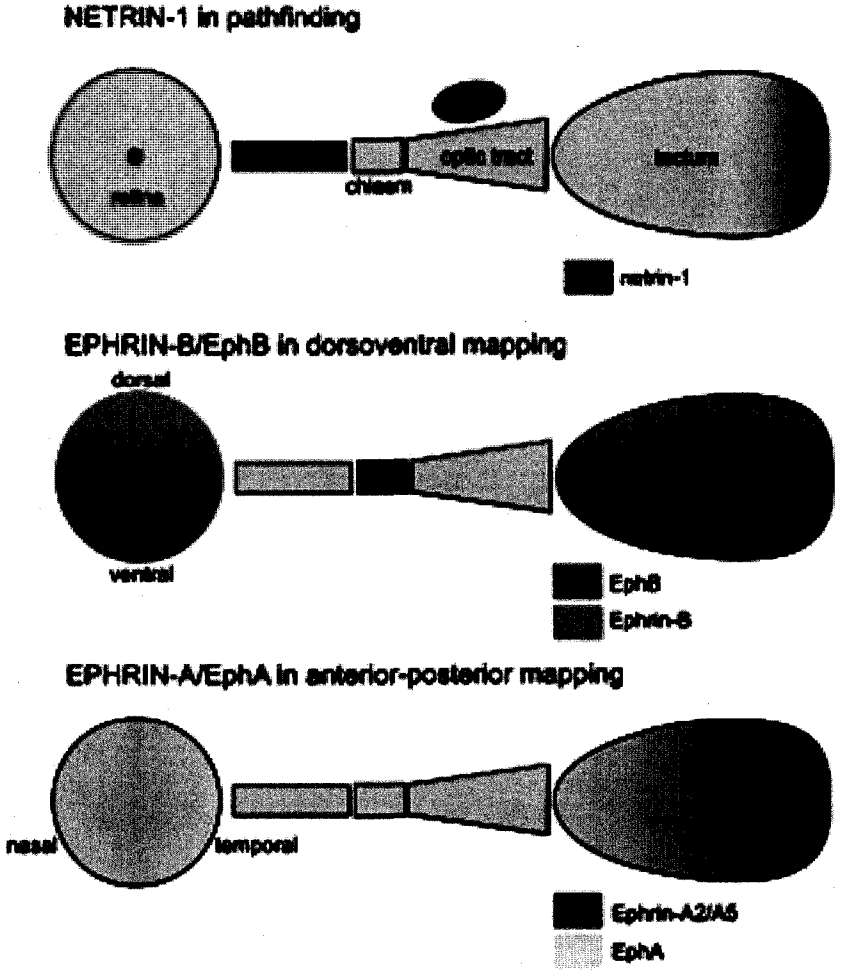
Diagram I.

A



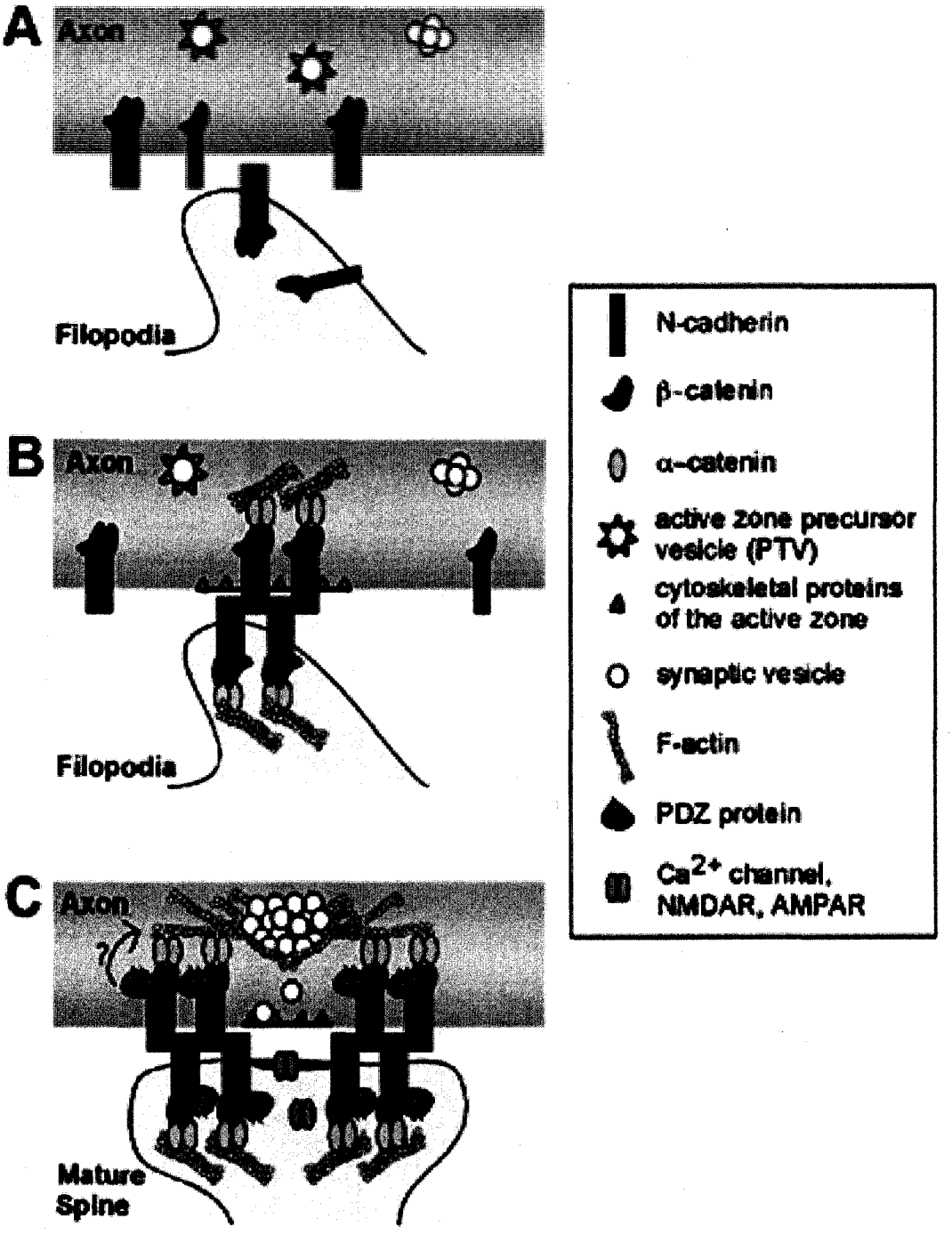
Adapted from (Mann et al., 2004)

B



Adapted from (Mann et al., 2004)

Diagram II.



Adapted from (Bamji, 2005)

General goal

N-cadherin is modulated by synaptic activity to create a potentially stronger bond between two cells at the synapse. Thus synapse maturation, including the recruitment and activation of cadherin adhesion at synaptic junctions, could lead to a shift in axonal branch dynamics favoring more stable synaptic contacts. In this master's thesis, we will assess whether **retinotectal axon branch stabilization during development is mediated by cadherin activity.**

Aims

• Aim 1: Test the role of N-cadherin in regulating RGC axon complexity

Previous time lapse *in vivo* imaging experiments in *Xenopus* demonstrated the relationship between mature synapses and localized sites of structural stability in RGC axons (Meyer & Smith, 2006; E. S. Ruthazer et al., 2006). N-cadherin is known to take part in the maturation of synapses; it was shown that presynaptic vesicles of axons were less abundant in dominant negative cadherin expressing cultured neurons (Tang et al., 1998; Togashi et al., 2002). In another study, a dominant negative form of β-catenin expressed in RGCs of *Xenopus* resulted in an atypical morphology (Elul, Kimes, Kohwi, & Reichardt, 2003). However, no direct link between cadherins and axonal arbor remodeling leading to map refinement has been directly established.

It has been shown that the expression of N-cadΔE results in poor axonal initiation, outgrowth, and pathfinding in the retinotectal system (Riehl et al., 1996). To investigate the role of N-cadherin in axonal branch stabilization, we used electroporation to transfect the RGCs at stage 43 and 44 of development when a large number of the retinotectal axon have already terminated in the tectum. Because these axons have already reached the tectum, the inability of RGC axons to arrive in the optic tectum due to the expression of a dominant negative N-cadherin early in developmental is bypassed. By performing both long (daily) and short (4hr) interval time-lapse imaging of cells co-expressing eGFP/N-cadΔE or eGFP only, the role of N-cadherin in axonal branch morphology is considered.

We first performed a daily time lapse *in vivo* imaging experiment to observe the elaboration of single axons co-expressing a cell filling eGFP fluorophore and a dominant negative N-cadherin construct (N-cadΔE). Post-hoc verification of expression was carried

out by wholemount immunohistochemistry. We predicted that due to an inability of N-cadherin to stabilize the RGC axon through postsynaptic interactions, the N-cadΔE cells would be less elaborate due to an inability to stabilize their branches. A second imaging was then performed where an 8-hour schedule of imaging was implemented – with an image every 4 hours. This experiment helped us understand the role of N-cadherin in axonal branch remodeling through a possible alteration in the dynamics of branch behavior. With N-cadherin signal hindered, we propose that either a decrease in branch additions or an increase in retractions could account for the failure of RGC axon arbors expressing the dominant negative N-cadherin to elaborate over time.

• Aim 2: To investigate how N-cadherin affects synaptic properties

As N-cadherin is know to be highly enriched in perisynaptic zones in the tectal neuropil and because interfering with N-cadherin signaling alters synaptic maturation, the synapse is the most likely site at which cadherin might extend its influence on axonal morphogenesis (Miskevich, Zhu, Ranscht, & Sanes, 1998; Yamagata & Sanes, 1995).

A synaptic vesicle protein tagged with a fluorophore, *synaptophysin-GFP (Syp-GFP)*, has effectively been used to characterize synapse number and the relative accumulation of synaptic vesicles thought to reflect synaptic maturity in axonal branches of *Xenopus* tadpoles (Meyer & Smith, 2006; E. S. Ruthazer et al., 2006). The intensity of the presynaptic Syp-GFP puncta then can be quantified. As the synapse matures there is an increase in Syp-GFP fluorescence due to accumulation in the number of presynaptic vesicles. This represents a powerful tool for exploring axonal arbor branching and growth with respect to synaptic location, density, size, and stability as observed in time-lapse images of developing RGC axons in the optic tectum.

Time-lapse imaging of single axonal branch projections co-expressing the dominant negative construct and synaptic marker synaptophysin has been carried out for this aim of the project. We combined the use of the Syp-GFP synaptic marker with short-interval imaging of structure using YFP to examine synapse formation in newly formed branches vs. older segments present early in the imaging sequence. This helped us distinguish older synapses that may have formed prior to electroporation from *newly* formed synaptic puncta, where we know the dominant-negative construct was expressing during its formation and maturation. Our study then discriminated synapse location, density, and maturity with respect to the addition or retraction of a certain branch or branch segment. Whether N-cadΔE disrupts the maturity or affects synaptogenesis in our system was investigated by observing the distribution and intensity of Syp-GFP puncta in the N-cadΔE group. Differences in arbor dynamics relative to the location of puncta would thus build on previous work asserting to the importance of mature synapses as sites of branch retraction. For this set of experiments we have tested the hypothesis that cadherin-mediated adhesion is a mechanism by which axonal arbor structure is *locally* stabilized at synaptic contacts. We used Syp-GFP to examine the relative locations of mature synaptic sites in RGC axons with respect to structural remodeling of lone axons over time. We predict that if N-cadherin contributes to the local axon stabilization at synaptic sites then N-cadΔE expressing axons will exhibit reduced stability at mature synaptic contacts.

Significance

Our aim is to find a direct link between N-cadherin and axonal stabilization leading to retinotopic map refinement. This fine-tuning of neural maps is a general theme found throughout the nervous system. We have begun to use the retinotectal projection as a model system for *in vivo* imaging with genetic manipulations to uncover a set of key molecular players in the fine-tuning and stabilization of retinotectal projections.

CHAPTER TWO.

Manuscript to be submitted to Journal of Neuroscience

Abstract

Retinal ganglion cell (RGC) axon arbors within the optic tectum are refined in development through a dynamic process of activity-dependent remodeling. The synaptic adhesion molecule N-cadherin is a candidate for mediating selective stabilization and elaboration of RGC axons due to its localization to perisynaptic sites and its modifiability by neural activity. RGCs of *Xenopus* tadpoles were co-transfected with plasmids encoding a dominant negative N-cadherin (N-cadΔE) and eGFP or eYFP to reveal cell morphology. Using two-photon *in vivo* time-lapse imaging, we found that axons expressing N-cadΔE became less elaborate than controls over three days of daily live imaging. To investigate the dynamic remodeling of the axonal arbor and concurrently visualize synaptic puncta labeled with synaptophysin-GFP (Syp-GFP), a shorter interval time-lapse imaging revealed a decrease in the fraction of branches that were stable, as well as an increase in the fraction of branches that were lost over eight hours of imaging in the N-cadΔE cells was observed. Furthermore, an analysis of Syp-GFP punctae confirmed previous results: retracting branch tips were halted at sites of relatively high Syp-GFP accumulation. In contrast, N-cadΔE axons failed to show a significant correlation between Syp-GFP puncta and sites where retracting branches paused, implying an impairment of synaptic sites (Syp-GFP puncta) to impede retraction of branches in N-cadΔE expressing cells. We conclude that N-cadherin participates in the stabilization of axonal branches at synaptic sites in the *Xenopus* retinotectal system.

Introduction

The development of the retinotectal projection in *Xenopus* tadpoles involves a retinotopic map initially driven by molecular cues that is further pruned and fine-tuned to form precise termination zones. This latter component of map formation is activity-dependent, as has been demonstrated by studies in which the disruption of normal retinal activity patterns leads to a degraded retinotectal map in the mouse (McLaughlin et al., 2003; Simon et al., 1992), the chick (Kobayashi et al., 1990), and the frog (Reh & Constantine-Paton, 1985).

Retinal ganglion cell axonal arbor refinement is thought to be dependent on synchronous neural activation of pre- and postsynaptic cells. This is mediated through synaptic activation of postsynaptic NMDA receptors, as blockade of tectal NMDArs has been shown to cause a disruption of the accurate mapping of axons in the tectum (Reh & Constantine-Paton, 1985). This emphasizes the importance of NMDAr activity in map formation and provides evidence that retrograde signaling pathways from post- to presynaptic cells is a key event during retinotectal map formation (Cline & Constantine-Paton, 1989; Schmidt et al., 2000).

RGC axons terminate in the tectal neuropil with an elaborately branched arbor, which is highly dynamic, extending some new branch tips into new territories while retracting others. It has been shown that mature synapses serve as anchoring sites where dynamic branches stabilize in their quest to optimize connectivity. Thus, the formation of stable synapses is required for activity-mediated fine-tuning of the retinotectal projection (Meyer & Smith, 2006; E. S. Ruthazer et al., 2006). However, the mechanism by which these contacts stabilize the axonal arbor is still poorly understood.

The synaptic molecule N-cadherin is a likely candidate for mediating the stabilization of RGC axons in development. N-cadherin is a single-pass transmembrane molecule with five extracellular repeat domains and an intracellular catenin-binding region (CBR). This configuration allows this protein to act as a transynaptic scaffolding unit as its CBR is thought to interact via catenin proteins to the actin cytoskeleton while its outermost EC-1 domain interacts homophilically with cadherin molecules on itself or on other cells in the extracellular environment (Shan et al., 2000). N-cadherin is expressed in RGCs and is found to be important for initiation, outgrowth, and axon guidance in the development (Matsunaga et al., 1988; Riehl et al., 1996). Once in the optic tectum, N-cadherin takes a punctate appearance and colocalizes with synaptic markers in RGCs (Miskevich et al., 1998; Uchida et al., 1996). There is some evidence cadherins may be important in synapse stabilization and maturation (Bozdagi et al., 2000; Huntley, 2002). Blocking N-cadherin function by expression of a dominant negative results in delayed synaptogenesis and synapse maturation (Bozdagi, Valcin, Poskanzer, Tanaka, & Benson, 2004). Hippocampal slice work reveals a decrease in excitatory post-synaptic potentials and the prevention of long-term potentiation induction in the presence of function blocking N-cadherin antibodies (Bozdagi et al., 2000; Jungling et al., 2006; Tang et al., 1998).

Not only does N-cadherin take part in the aforementioned events, but it does so by changing morphologies from a monomer to a dimer or multimer upon NMDAr activation and neural depolarization (Tanaka et al., 2000). Upon activation, this molecule becomes resistant to proteolysis which may increase its ability to persistently link synaptic sites. Furthermore, the α/β-catenin complex is known to aggregate to synaptic sites upon NMDAr activation, and may play a role in the dimerization of N-cadherin (Imamura et

al., 1999; Murase et al., 2002). The formation of stable synapses thus appears be linked to an activity-dependent dimerization of N-cadherin that would in turn link the F-actin cytoskeleton to extracellular adhesion.

In this study, we used *in vivo* time-lapse imaging of the RGC axonal projection into the optic tectum to investigate the role of cadherins in the developmental refinement of the retinotectal projection. A dominant negative N-cadherin, N-cadΔE, was expressed in individual RGCs in developing *Xenopus* tadpoles along with eGFP or eYFP fluorophores to visualize RGCs. A synaptophysin-GFP (Syp-GFP) construct coexpressed with cell filling eYFP was also used to reveal synaptic puncta distributions, representing putative presynaptic terminals. It was found that N-cadΔE expressing cells became less elaborate than control axons over three days of imaging. Shorter interval imaging revealed an decrease in the relative stability of the N-cadΔE expressing arbors. Finally, we demonstrated that this change in dynamics could be attributed to the fact that individual synaptic sites, which normally function as sites of increased arbor stability, become less effective at halting axonal branch retraction when cadherin function is inhibited. It is concluded that N-cadherin plays a key role in stabilizing synapses in the developing RGC axons, hence regulating overall arbor structure and refinement.

Materials and methods

Retinal Ganglion cell transfection in Xenopus tadpoles.

RGCs were transfected by electroporation (E. S. Ruthazer, Haas, K., Javaherian, A., Jensen, K.R., Sin, W.C. and Cline, H., 2005). Briefly, stage 43-44 (Nieuwkoop PD & Faber, 1956) *Xenopus* tadpoles reared in identical pre-manipulation conditions, were anesthetized by immersion in 0.02% MS-222 (Sigma, St. Louis, MO) and placed on a wet Kimwipe wiper under a dissecting microscope. The tip of a glass micropipette was inserted between the lens and the neural retina to deliver plasmid DNA solution (~1- 6 μg/μL in water mixed with Fast Green for visualization) by pressure injection. Current was then passed across the eye through platinum electrodes placed on opposite sides of the eye. A voltage pulse of 34-38 V with a duration of 1.6ms in 2-5 pulses was generated using a GRASS SD9 stimulator (Grass SD9; Grass Instruments, Quincy, MA) with a 3μF capacitor placed in parallel to generate exponential decay of current intensity over time. The N-cadΔE plasmid was cotransfected with enhanced-Green Fluorescent Protein (eGFP) and enhanced-Yellow Fluorescent Protein (eYFP) in a ratio of 3:1 to ensure cotransfection of N-cadΔE into most visualized cells. For synapse visualization, a conjugated synaptophysin-GFP was cotransfected with eYFP - in a ratio of 1:1.

Twelve different types of RGCs have been described in the *Xenopus* retina (Straznicky & Straznicky, 1988). Our method does not allow us to distinguish between them based on axonal morphologies, which may have contributed additional variability in our dataset.

Immunohistochemistry

Xenopus tadpoles expressing N-cadΔE, which is *myc*-tagged, in RGC axons were fixed in 4% paraformaldehyde overnight at 4°C. The animals were rinsed in 0.1M phosphate buffer and their brains were dissected and pre-incubated in blocking solution consisting of 0.03% triton-X100 (AMD Gibbstwon, NJ) and 5% normal goat serum (SIGMA St-Louis, MO) in 0.1M phosphate buffer. The brains were then incubated at 4°C overnight in an anti-*myc* 1° antibody (mouse monoclonal at 1:100 dilution; Invitrogen, Eugene, OR). The tissue was washed repeatedly and left overnight in blocking solution, after which they were stained with an Alexa-555 Goat anti-Mouse IgG (1:1000 dilution; Invitrogen, Eugene, OR). The brains were placed in a specially designed Sylgard chamber, and imaged with the use of a two-photon microscope for post-hoc analysis of N-cadΔE expression. Only axons testing positive for *myc* were included in our experimental groups analysis.

Two-photon imaging

After 48-72 hours, tadpoles were screened for fluorophore-expressing single axons projecting to the optic tectum using mercury-lamp epifluorescence with a 20X (numerical aperture, 0.5; Olympus) objective. To limit harmful exposure to excitation light, illumination was kept to a minimum. Tadpoles were first anesthetized by immersion in 0.02% MS-222, then mounted in a Sylgard chamber specially designed to immobilize the animals by contouring their body under a cover glass. Images were collected at regular intervals from 4-24 hrs over a maximum of three days. Z-series were collected at 1μm spacing with a zoom of 1.5-2X using a 60X water immersion objective (numerical aperture, 1.1; Olympus). A Mai Tai femtosecond pulsed Titanium Sapphire Laser

(Spectra Physics) set at 910 nm (eGFP, mCherry) or 990 nm (eYFP, Alexa 555) wavelengths provided multiphoton fluorescence excitation. Fluorescence emission was collected using multialkali photomultiplier tubes through the following custom two-photon blocked bandpass emission filter sets from Chroma (Rockingham, VT) : eGFP/mCherry: HQ525/50m, 560dclp, HQ620/60m; CFP/eYFP: HQ480/40m, 510dclp, HQ540/40m.

Reconstruction of retinotectal axonal projections

Three-dimensional axon reconstruction and quantification were performed with the use of Object Image and Imaris (Bitplane) software. For daily imaging Imaris was used to manually trace the object in three dimensions; results of segment lengths and branchtips were gathered automatically. For dynamics and punctum analysis, Object Image (http://simon.bio.uva.nl/Object-Image/object-image.html) was used to follow individual branches across the time points.

Calculation of punctum intensity in time-lapse images

The arbors of axons co-expressing Syp-GFP and YFP were reconstructed in three dimensions using Object-Image based on the YFP channel image. This reconstruction was then overlaid onto the Syp-GFP stack. Puncta along each axonal branch were identified and measured using previously described custom Object-Image macros (Ruthazer et al., 2006). To correct for differential levels of plasmid expression, the fluorescence intensities of all Syp-GFP puncta in a single axon were normalized to the 80%-ile brightest punctum in each arbor.

Results

N-cadherin distribution in living Xenopus retinotectal axons

The cell adhesion molecule N-cadherin has been reported to become increasingly localized at synaptic sites over development (Bozdagi et al., 2004; Jontes et al., 2004). Our previous electron micrographic analysis demonstrated that exogenously expressed tagged synaptophysin protein accumulates selectively at morphologically identifiable presynaptic sites (Ruthazer et al., 2006). Therefore to confirm the synaptic localization of N-cadherin in the developing *Xenopus* retinotectal system, RGCs of stage 45 tadpoles were co-transfected with *Xenopus* N-cadherin fused with eGFP (XNcad-GFP) along with a synaptic vesicle protein fused with the mCherry fluorophore (Syp-mCherry) (Fig. 1 ***A-C***). XNcad-*GFP* and Syp-mCherry have clearly punctate and largely overlapping distributions within RGC axon terminals, demonstrating that N-cadherin is highly enriched at synaptic sites in *Xenopus* RGC axon terminals during development.

N-cadherin signaling mediates RGC axon complexity

To investigate the role of N-cadherin in arbor refinement, we performed *in vivo* two-photon time-lapse imaging of RGC axons over a three-day period. As cadherins have been shown to be necessary for early RGC axon outgrowth and guidance prior to reaching the optic tectum, we used electroporation to express a dominant negative form of N-cadherin (N-cadΔE) in postmitotic RGCs of animals (in stages 43-44) which had already extended axons into the tectal neuropil and had begun the process of synaptogenesis. Axons were electroporated with a plasmid expressing eGFP and the experimental group was coelectroporated with N-cadΔE Because retinal electroporation

of two plasmids yields approximately 70% of cells with high levels of coexpression of both plasmids, we performed posthoc immunohistochemistry against the myc tag on the N-cadΔE construct to confirm coexpression in all experimental cases (Fig. 2 ***B-E***). Cases where coexpression could not be confirmed were discarded. The axonal arbors of RGCs expressing the N-cadΔE construct did not show obvious gross abnormalities of morphology or growth. Both the GFP control (Fig. 2 ***F***) and the N-cadΔE/GFP groups (Fig. 2 ***G***) demonstrated dynamic rearrangements of their arbors over three days. The mean overall branch lengths, calculated from the first branch bifurcation of each axon arbor (Fig. 2 ***H***) of the dominant negative expressing axons did not differ significantly from those of the eGFP expressing cells (Fig. 2 ***I***). However, when arbor complexity was quantified by counting the number of branchtips in each axonal arbor (Fig 2. ***J***), branchtip number in the N-cadΔE expressing axons was found to be reduced compared with control eGFP expressing cells by the third day of imaging (Fig. 2 ***K***). Indeed, by the third day of imaging the N-cadΔE expressing arbors were no more complex than they had been on the first day of imaging. This finding suggests that N-cadherin function, while not necessary for overall RGC axon arbor growth in the tectum, mediates arbor elaboration and branch accumulation over development.

Rates of axonal branch addition and stabilization affected by N-cadΔE

The decreased branch complexity of N-cadΔE expressing axons could be the result either of an impairment in the ability of the neuron to add new branches, or to the decreased stability and consequent retraction of existing branches. A short interval imaging protocol was used to investigate the dynamic branching behaviors of axons on the equivalent of day 3 in the daily imaging protocol when the effect of N-cadΔE expression

was greatest (Fig. 3 ***A***). We again collected images at three time points, this time separated by four hours (Fig. 3 ***B*** & ***C***). The relative proportion of added, lost and stable branch tips were quantified. The N-cadΔE expressing axons exhibited an increase in the proportion of branch tips that were eliminated over the 8 hour imaging period, suggesting that these axons do not form as many long-lasting branches as controls (Fig 3 ***D***). Consistent with this finding, we also observed a smaller proportion of stable branch tips, present throughout the full 8 hours, in the N-cadΔE group (Fig. 3 ***D***). Furthermore, there was no decrease in the rate of branch addition in the N-cadΔE neurons, supporting the idea of decreased branch stability rather than an impairment of branch addition.

N-cadΔE does not affect the distribution or maturity of synapses in RGCs

It has been demonstrated previously that the axonal arbor is preferentially stabilized at mature synaptic sites. The localization of N-cadherin in the perisynaptic region raises the possibility that axonal stabilization at synapses is mediated by cadherin-mediated adhesion and signaling at the synapse. This could be accomplished either by cadherin-mediated facilitation of synaptogenesis or synapse maturation leading to more stable arbors, or by the direct adhesive role of cadherin at existing synapses.

To test for an influence of cadherin signaling on synapse formation and maturation, we co-expressed Syp-GFP, which labels accumulations of synaptic vesicles likely to be presynaptic terminals (Ruthazer et al., 2006), along with YFP to reveal morphology. The Syp-GFP fluorescence intensity, reflecting synaptic maturity, and the density of putative synaptic puncta per 100μm length of axonal branch were measured along newly formed branches that were not present in the first image. Measuring new branch tips assured that the synapses being analyzed formed after transfection with the dominant negative

cadherin construct in the experimental group. New branch tips were compared to the stable skeleton branches at the center of the arbor of the same axons; synapses on these branches are likely to have formed prior to transfection (Fig 4. ***A***). Analysis of distribution and the intensities of the Syp-GFP puncta as a ratio of new branches vs. old skeleton branches across the N-cadΔE and control groups did not yield any significant differences (Fig. 4 ***B***). Hence we could not conclude based on these observations that N-cadherin function interferes with synaptogenesis or the accumulation of synaptic vesicles at synaptic sites. We therefore next examined whether cadherin participates in the stabilization of the arbor at existing synapses.

N-cadΔE reduces branch stabilization at mature synaptic sites

We previously established that there is a spatial relationship between intensely labeled Syp-GFP puncta and the point at which axonal branch tips are stabilized against retraction (Ruthazer et al., 2006). Figure 5 ***A***, and ***B***, show examples of partially retracting branches in control and N-cadΔE expressing axons over 4 hrs. In the control group (Fig. 5 ***A***), it is clear that the two retracting branches halt their withdrawal at puncta of relatively bright Syp-GFP expression. On the other hand, the dominant negative N-cadherin expressing axon branch can be seen retracting beyond a bright Syp-GFP expressing puncta (Fig. 5 ***B***). Retractions resulting in the elimination of a mature Syp-GFP punctum are extremely rare in control axons. This snapshot of branch dynamics is further revealed by scatterplots (Fig 5 D, E) which show the locations of all the individual Syp-GFP puncta on branches from control and N-cadΔE expressing axons that retracted over 4 hr of imaging, categorized by Syp-GFP brightness. This is quantified in Figure 5 ***F*** which compares the normalized intensities of Syp-GFP puncta that were: a) in the

segments of the retracting branches that were spared in the retraction, b) within 1μm of the point at which the retraction halted at the second time point 4 hr later (*retraction point*; diagonal line in Fig. 5 ***D*** & ***E***), or c) lost during the branch retraction. In control axons there was a highly significant difference between punctum intensities in the lost part of the arbor compared with puncta at the retraction point (Fig. 5 ***F***). This confirms our earlier report that branch retractions are slowed or halted at mature synaptic sites. Puncta at the retraction point are also more intense than in the rest of the spared branch, indicating that less mature synaptic sites, as can be found throughout the axonal arbor, are not as effective as mature sites for halting branch retractions. Although there appears to be a trend toward N-cadΔE expressing axons having higher intensity puncta at their retraction points, this was not significant, largely because of the substantially larger fraction of mature puncta (> 40 relative intensity) in the lost segments of the branches (Fig. 5 ***G***). We conclude that normal cadherin function is important for conferring structural stability to axon arbors at existing presynaptic sites.

Discussion

This study investigated the role of cadherin in the refinement of RGC axon arbors in the optic tectum of the *Xenopus* tadpoles. Inhibition of cadherin function prevents the normal increase in RGC arbor branch number that occurs as the axon arbors elaborate within the tectum. This was in part explained by the relative decrease in the number of stable branches and the increase in the number of lost branches over the imaging period observed in the N-cadΔE axons compared to control cells. Additionally, it has been suggested that synaptic puncta create a framework of stable sites along skeletal branches in axonal arbors, from which exploratory branches extend forward but generally do not retract past. This model permits arbor remodeling while keeping a portion of the arbor 'anchored' by mature puncta (E. S. Ruthazer et al., 2006). Using Syp-GFP to visualize puncta in both control and N-cadΔE groups expressing groups, we found that compared to control axons, mature Syp-GFP puncta were more often found in parts of the arbor that were eliminated by branch retraction and less often at the site where the retraction halted. Therefore, N-cadherin at synapses appears to contribute to their ability to stabilize the arbor locally. Figure 6 schematizes our interpretation of the relationship between synaptic sites and arbor remodeling. It also incorporates a hypothetical role of the postsynaptic assembly, as it has been raised that NMDAr mediated activation in postsynaptic cell is a way for the postsynaptic environment to detect (activity) correlation, which is instrumental in axonal map refinement (Schmidt, 2004). Our findings demonstrate that presynaptic N-cadherin function is necessary in the regulation of RGC axon stabilization.

Even if both pre- and postsynaptic activities contribute to the refinement of the retinotectal projection, their respective responses to neural activity blockade are different. The rate of dynamic rearrangements in axons increases with activity blockade whereas tectal neuron dendrites show reduced dynamics (Rajan, Witte, & Cline, 1999). Although we did see an increase in the number of lost branches in the N-cadΔE axons over controls, an increase in additional branches did not arise – this suggests that cadherin probably functions downstream of synaptic activity, effecting some consequences of activity manipulation like the degree of branch stabilization but not others, such as the rate of branch addition.

Although it has been shown that N-cadherin is found at putative synaptic sites, and that the expression of a dominant negative cadherin delays synaptogenesis with a decreasing synapse size in hippocampal neurons (Bozdagi et al., 2004); recent evidence suggests N-cadherin's role in most aspects of synapse formation is only transient and readily compensated for by the cell (Latefi, 2006). This is consistent with our finding that N-cadΔE expression did not produce changes in synapse density or maturation detectible by our methods; it further supports a model in which cadherin function at existing synapses might be influenced by neural activity rather than one in which cadherin's ability to transiently modulate synaptogenesis is responsible for its effects on arbor elaboration.

The role of postsynaptic activity in the refinement of the presynaptic retinotectal axons has been noted in a number of studies where NMDAr blockade was targeted to postsynaptic cells (Cline & Constantine-Paton, 1989; E. S. Ruthazer et al., 2003; Schmidt et al., 2000). These and other experiments support the existence of retrograde signaling from post- to presynaptic cells (Schmidt, 2004; Zou & Cline, 1996). N-cadherin

function is also affected by changes in neural activation of postsynaptic NMDArs : It has been shown that depolarization of hippocampal neurons drives the dimerization of N-cadherin into a functional *cis*-dimer (on the same synaptic plane), likely enabling the formation of transsynaptic *trans* dimers . This process effect also renders N-cadherin less susceptible to proteolysis (Takeichi, 2007). Thus transsynaptic N-cadherin adhesion and signaling may contribute to the retrograde signaling that stabilizes the presynaptic axonal arbor.

Translocation of ß–catenin to cadherin loaded synaptic sites is linked to dendritic depolarization and could yield a link to increased presynaptic maturation. It has been shown in axons that ß-catenin interacts intracellularly, linking the actin cytoskeleton with N-cadherins and other PDZ containing proteins and scaffolding proteins while also recruiting vesicles to synaptic sites (Bamji, 2005; Bamji et al., 2003). In a series of specific deletions, ß-catenin domains required for cadherin, alpha-catenin binding and PDZ protein binding show drastic morphological differences in the elaboration of RGC projections in the optic tectum of *Xenopus* tadpoles (Elul et al., 2003). A parallel between morphologies in the ß-catenin deletions and our N-cadΔE axons is seen in this inability to elaborate axonal projections forming a complex arbor *in vivo*. This transynaptic cytoskeletal connection through an N-cadherin/catenin may have role defining synaptic zones and aid not only in their formation but in their maintenance (Uchida et al., 1996).

Postsynaptic glutamate receptor subunits have been shown to interact with N-cadherin. It was shown that the GluR2 subunit interacts directly with the cis/trans configuration of N-cadherin in mouse hippocampal preparations. A link between maturation and strengths of synaptic sites due to AMPA receptor presence is then linked to interactions with N-cadherin signaling (Saglietti et al., 2007). A dominant negative N-

cadherin expressed in chick horizontal cells in the retina impaired GluR4 accumulation on their postsynaptic target sites (Tanabe et al., 2006). In our system, N-cadΔE may interfere with the N-cadherin/AMPA receptor interaction at synaptic sites. Although it is possible that AMPA trafficking in the presence of N-cadΔE is abnormal, we can speculate that it is the presynaptic cells expressing N-cadΔE will lack normal N-cadherin/AMPA interactions. (Tanabe et al., 2006)

It remains to be demonstrated that N-cadherin function and signaling at the retinotectal synapse can be influenced by visual experience. Since activity drives cadherin dimerization in cultures (Tanaka et al., 2000), this can be further investigated by an *in vivo* analysis of the levels of homodimer/multidimer of N-cadherin upon visual activation. This can be achieved by conjugating the X-Ncad protein with a FRET-pair of fluorophores (CFP + YFP or mCherry + GFP). An increase in the acceptor fluorescence could be used to indicate a change in conformation from an N-cadherin monomer to a multimer in response to enhanced visual stimulation, or the reversal of this process by activity blockade.

Similarly one could incorporate the methodology used in Ruthazer et al. (2006), to observe the effect that N-cadΔE has on visual stimulation-induced changes in arbor dynamics and branch stabilization at synaptic sites. It can be speculated that N-cadΔE expressing axons would be less responsive to activity if trans-synaptic signaling through cadherins is occurring. Even more compelling would be to reduce cadherin function in the postsynaptic cells and observe the impact on presynaptic axons. However, current gene transfection technology in *Xenopus* does not yet permit efficient expression of constructs in the complete set of postsynaptic target neurons as would be required for this experiment.

Our work indicates that N-cadherin is a key player in the elaboration of axonal arbors in the developing retinotectal system of *Xenopus* tadpoles. It proposes a role for N-cadherin as a scaffolding molecule that tethers the pre- and postsynaptic partners, allowing the synapse to become a site of stabilization.

Figure captions

Figure 1. Co-expression of XNcad-GFP with SYP-mCherry in RGC axonal projections in tectum of stage 45 tadpole. ***A***, expression of *Xenopus* N-cadherin tagged with GFP. ***B***, Synpatophysin-mCherry expression. ***C***, clearly punctate and overlapping distributions of XNcad-GFP and Syp-mCherry demonstrate presence of N-cadherin at synaptic sites during development. Scale bar, 10 μm.

Figure 2. N-cadherin plays a role in the elaboration of axonal arbors in RGCs. ***A***, timeline of daily imaging experiment. Stage 43-44 tadpoles are electroporated with eGFP or N-cadΔE/eGFP plasmids and allowed a few days to express the DNA. Animals with single axons projecting onto the tectum were imaged once daily for three days. After the imaging experiment, post-hoc immunohistochemistry against *myc* was performed to confirm expression of the N-cadΔE. ***B***, live image of eGFP in a N-cadΔE expressing axon on the last day of imaging, ***C*** image of eGFP in same axon after fixation; ***D*** anti-*myc* immunostaining (Alexa 555 secondary antibody), ***E*** merged image confirms N-cadΔE expression. Images of representative control (***F***) and N-cadΔE expressing (***G***) axons, taken once daily over 3 days. RGC axon arbors were reconstructed and quantified and using Imaris software (***H*** & ***J***). N=9 control and 10 N-cadΔE axons, * $p < 0.05$ two-tailed Student's t-test. Error bars indicate SE. Scale bar, 10 μm.

Figure 3. Rates of axonal branch addition and stabilization affected by N-cadΔE

A, timeline of short term imaging experiment. Tadpoles are electroporated at Stage 43-44 then screened and imaged on day 3 (relative to daily imaging experiment) for an image every 4hrs for a total of 3 images. A post-hoc immunohistochemistry is also performed as shown in Fig. 2. ***B*** & ***C***, representative control and N-cadΔE expressing cells with reconstructions below each image (Object Image). ***D***, ratios of added, lost, or stable branches vs. total # branches in each axonal branch were averaged across each condition. A two-tailed Student t-test was performed to distinguish differences between the means of each added, lost, stable branch ratio between the control and the N-cadΔE conditions. N-cadΔE (N=8) expressing cells had a greater number of lost branches vs. control (N=9) expressing cells ($p < 0.05$), while control axons had more stable branches than N-cadΔE axons ($p < 0.05$). Error bars indicate SE. Scale bar, 10 μm.

Figure 4. Punctum intensity and density distributions are similar in control and N-cadΔE groups. ***A***, A diagram representing *new* branches in red on a previously on a pre-existing black arbor. The intensity density of puncta along these newly added branches are 'found' and quantified by specifically written Object Image macros. ***B***, a two-tailed Student t-test was performed to distinguish differences in intensity and density ratios of puncta found on old branches vs. new branches amongst the control and N-cadΔE groups. Both intensity and density were found to be similar (N=7 control and 5 for N-cadΔE ($p > 0.05$)). Error bars indicate SE.

Figure 5. Branches retract to brighter Syp puncta in controls than N-cadΔE. ***A***, example of control axonal branch retracting up to bright Syp-labeled punctum; ***B***, an N-cadΔE branch retracting past sites of bright puncta. *Yellow* arrows represent Syp puncta and *white* arrow branchtips. ***C***, axonal branch retraction schematic, yellow dots represent Syp-GFP synaptic puncta. The black '**p**' bars represent the distance from punctum to branchtip, and the red '**r**' arrow is a measurement of the retraction distance of the branch. ***D*** & ***E***, scatterplot measurements of puncta along retracting branches from control (***D***) (N=8 cells, 1 per animal) and N-cadΔE (***E***) (N=8 cells, 1 per animal) groups, red dotted line depicts the area within 1μm of the 'retraction point' along the branch. Each line of color-coded punctum (color is intensity level) represents punctum across one branch. To the left of the 'retraction point' (red dotted line) are the spared punctum along the branch, while the ones to the right the lost punctum present on the 'lost' portion of the branch. ***F***, comparison of intensity (Syp-eGFP expression) of punctum by location; spared (N=733 control, N=746 N-cadΔE), within 1 μm of the retraction point (N=112 control, N=149 N-cadΔE), and lost (N=350 control, N=493 N-cadΔE) areas in control and N-cadΔE axons expressing Syp-eGFP and YFP. ***G***, proportion of puncta by intensity relative to their initial location before retraction; on spared, within 1μm of the retraction point in the lost portion of the retracting branch. * $p < 0.05$, *** $p < 0.001$; One-way ANOVA, followed by Bonferroni multiple comparisons analysis. Error bars indicate SE. Scale bar, 5 μm.

Figure 6. Illustration of the stabilizing role of N-cadherin at the synapse. ***A***, axonal branch extending past a mature synapse with a postsynaptic dendrite, glutamate receptors, and perisynaptic N-cadherin. ***B***, illustrates a branch retraction in the control group up to a mature synapse. ***C*** (control), retraction of axonal branch near – but not past – a mature synapse and further extension in another direction, while ***D*** (N-cadΔE) illustrates the dominant-negative axonal branch retracting past mature synapse due to N-cadΔE presence at presynaptic site.

Figure 1.

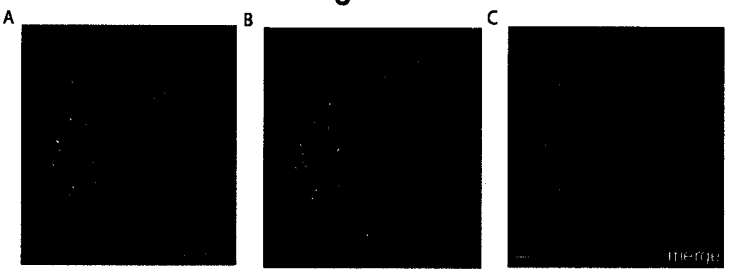
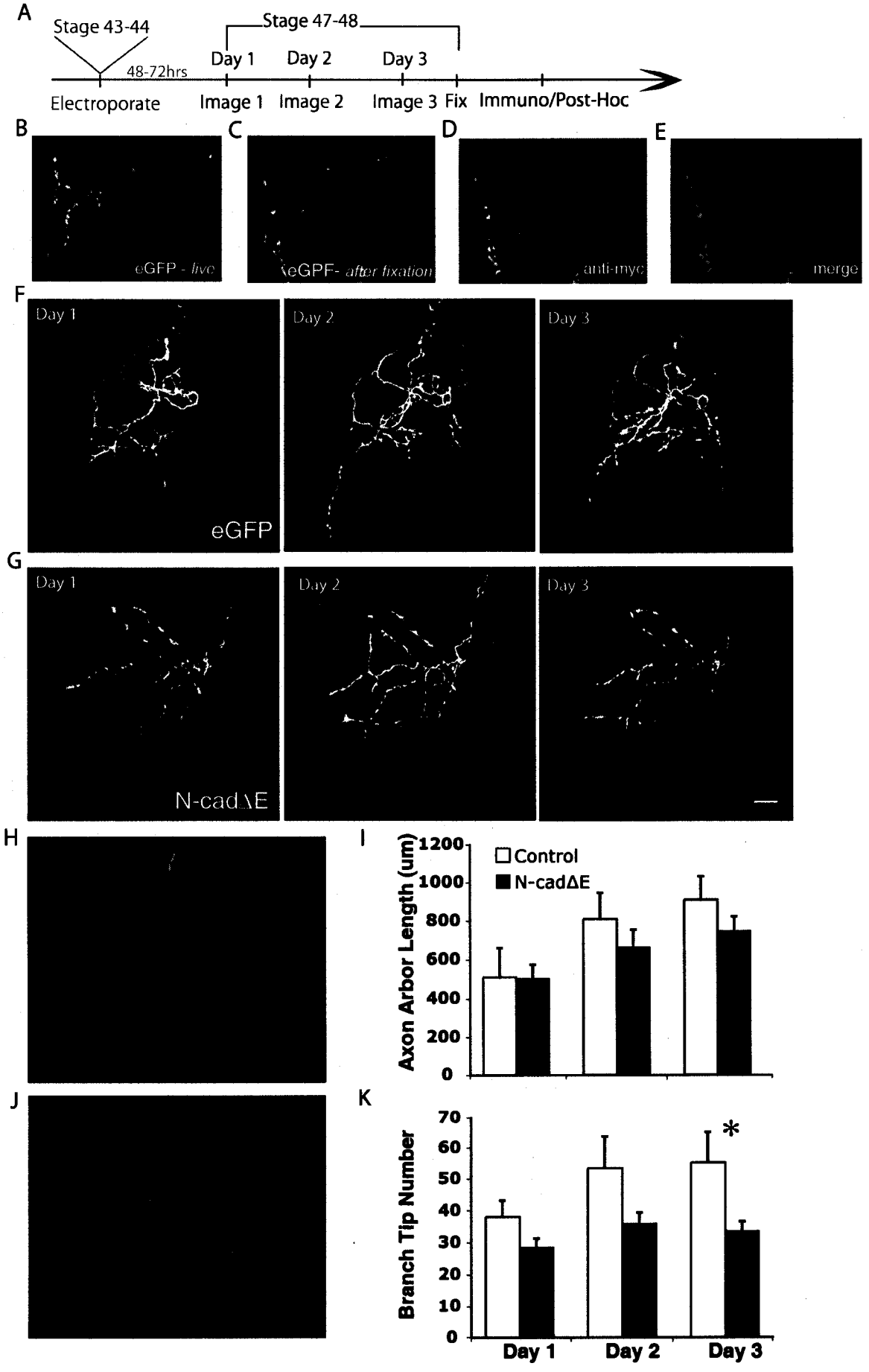


Figure 2.


A
Stage 43-44
Stage 47-48
48-72hrs
Day 1
Day 2
Day 3
Electroporate
Image 1
Image 2
Image 3
Fix
Immuno/Post-Hoc
B
eGFP - live
C
eGPF- after fixation
D
anti-myc
E
merge
F
Day 1
Day 2
Day 3
eGFP
G
Day 1
Day 2
Day 3
N-cadΔE
H
J
I
Control
N-cadΔE
Axon Arbor Length (um)
1200
1000
800
600
400
200
0
K
Branch Tip Number
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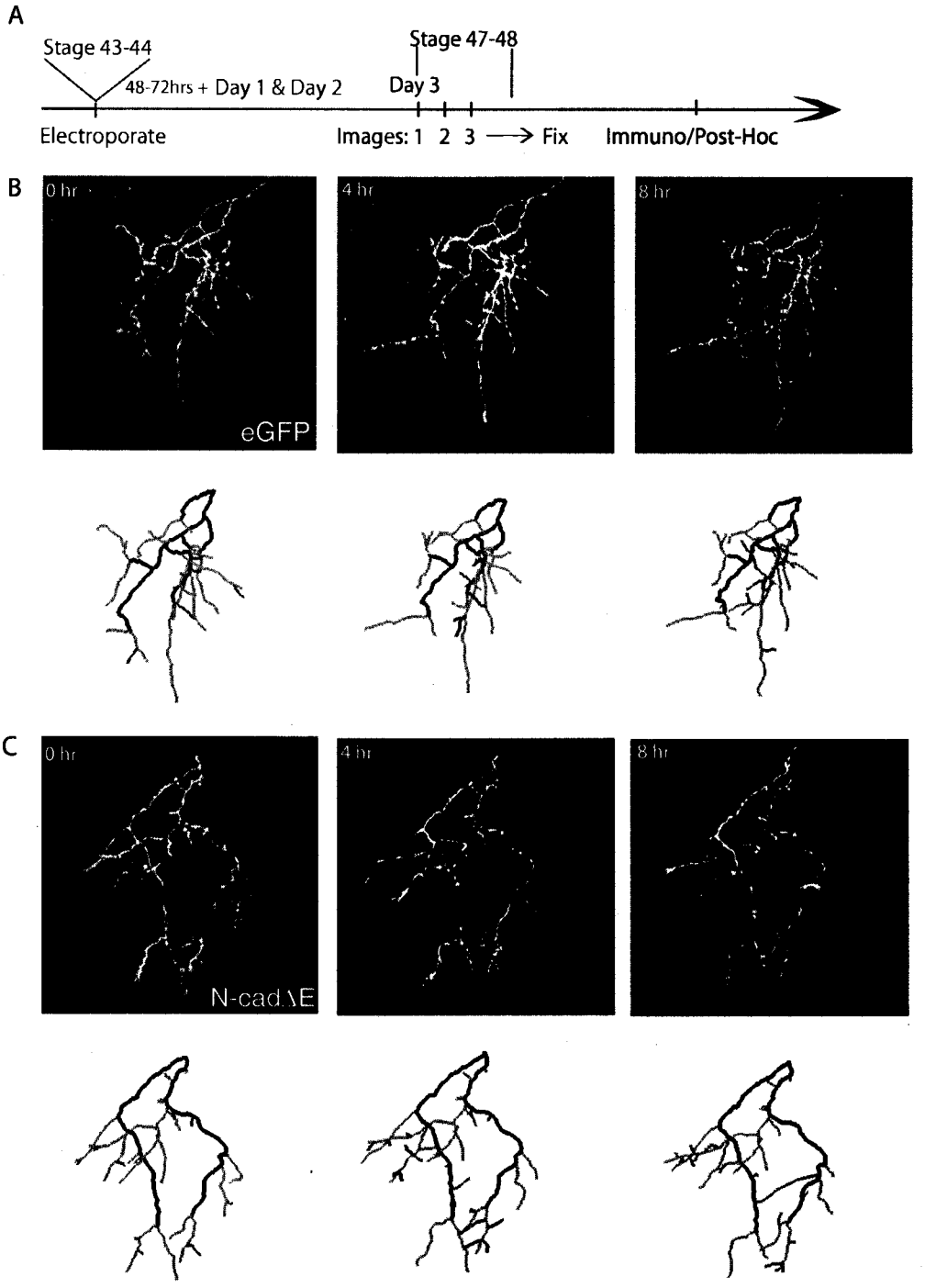
60
50
40
30
20
10
0
*
Day 1
Day 2
Day 3

Figure 3.


A
Stage 43-44
48-72hrs + Day 1 & Day 2
Stage 47-48
Day 3
Electroporate
Images: 1 2 3 → Fix
Immuno/Post-Hoc
B
0 hr
4 hr
8 hr
eGFP
C
0 hr
4 hr
8 hr
N-cadΔE
D
Fraction of Branch Tips
60%
50%
40%
30%
20%
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Control
N-cadΔE
*
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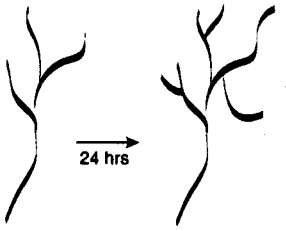
added
lost
stable
legend
lost
added
transient
stable
skeleton

Figure 4.

A


24 hrs

B

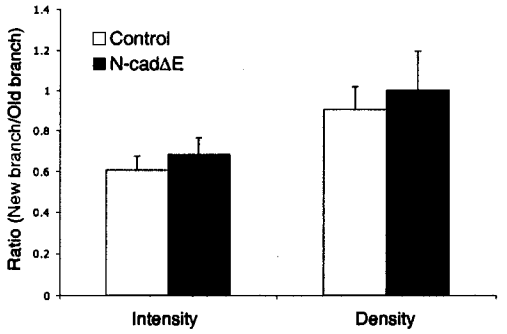

Ratio (New branch/Old branch)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
N-cadΔE
Intensity
Density

Figure 6.

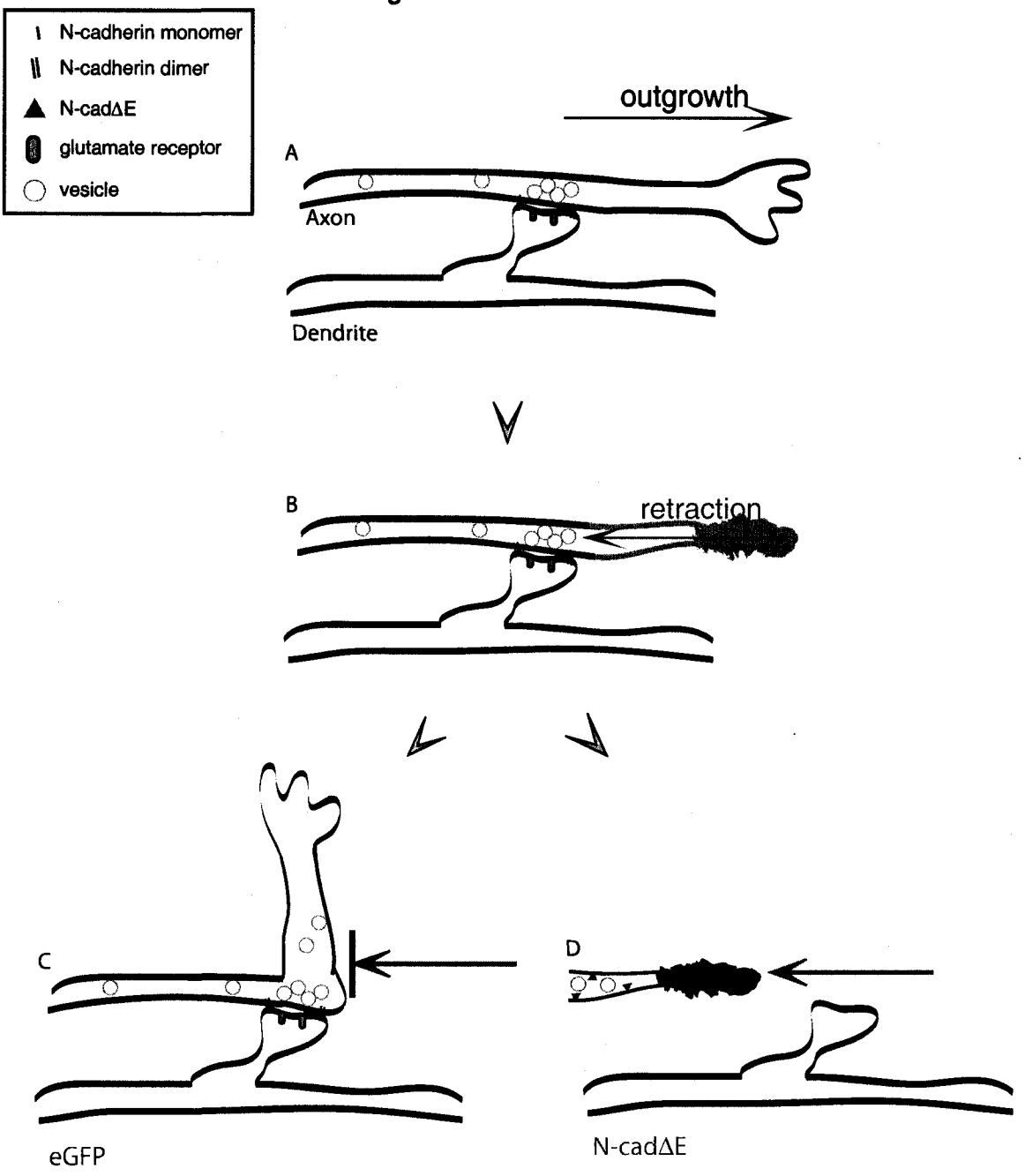
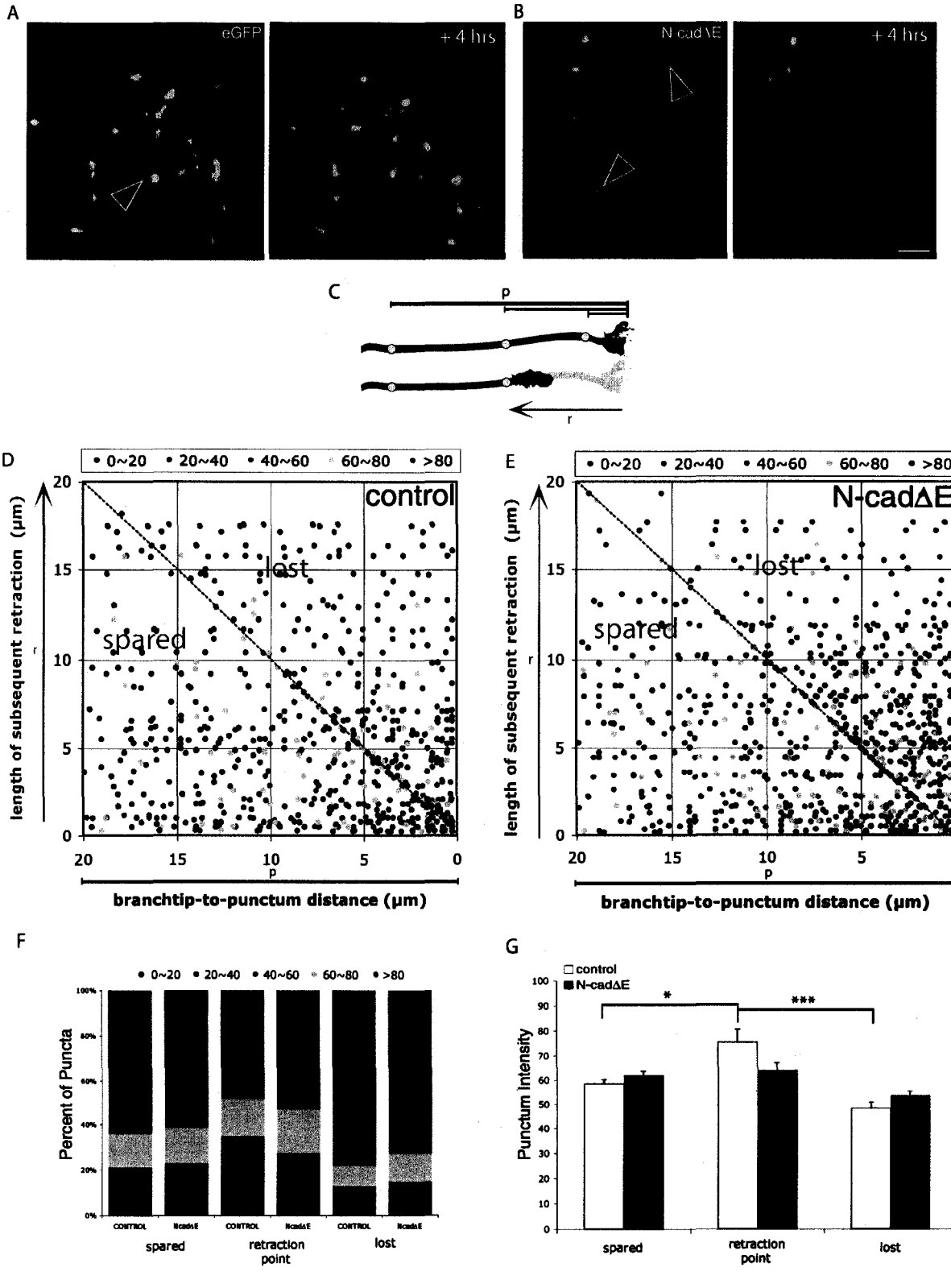

N-cadherin monomer
N-cadherin dimer
N-cadΔE
glutamate receptor
vesicle
outgrowth
A
Axon
Dendrite
B
retraction
C
eGFP
D
N-cadΔE

Figure 5.


A
eGFP
+4 hrs
B
N-cadΔE
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• 0~20 • 20~40 • 40~60 • 60~80 • >80
control
lost
spared
length of subsequent retraction (μm)
branchtip-to-punctum distance (μm)
E
• 0~20 • 20~40 • 40~60 • 60~80 • >80
N-cadΔE
lost
spared
length of subsequent retraction (μm)
branchtip-to-punctum distance (μm)
F
• 0~20 • 20~40 • 40~60 • 60~80 • >80
Percent of Puncta
CONTROL
NcadΔE
spared
retraction point
lost
G
control
N-cadΔE
*
***
Punctum Intensity
spared
retraction point
lost

CHAPTER THREE.

Concluding remarks and appendices

Concluding remarks

In our study we used a dominant-negative N-cadherin plasmid to qualitatively and quantitatively measure the impact of N-cadherin in the developing RGC axons. Although we do not have a complete knockdown of N-cadherin in our cells, we successfully fill (data not shown) the axonal arbor with our N-cadΔE construct. A morpholino knockdown would be another useful tool to eliminate (although not completely again) endogenous N-cadherin. However, we cannot create a knockdown before RGCs have reached the tectum so as not to impede outgrowth (Riehl et al., 1996). Furthermore, we are in the experimental phase of implementing morpholino knockdown in our lab and need to perfect our protocol. An RNAi experiment would also be something worth investigating for targeting N-cadherin mRNA, but the ability to employ it in *Xenopus* is unfortunately quite challenging.

Nevertheless, the differences seen in our study are genuine even though endogenous N-cadherin proteins are expressed in cells included in our N-cadΔE groups. The dominant negative N-cadherin is driven by an efficient CMV promoter creating an abundance of N-cadΔE, which competes against endogenous N-cadherin for intracellular binding partners.

Our results have successfully shown that N-cadherin is involved in the elaboration of RGC axons by increasing the ability of existing presynaptic sites to stabilize the arbor. A dominant negative N-cadherin may disassemble adhesive complexes at synapses, eliminating the intended structural stabilization of branches at synaptic sites.

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Appendices