CAMP PROMOTES RETINAL MIDLINE CROSSING AT THE ZEBRAFISH OPTIC CHIASM

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ABSTRACT

CAMP PROMOTES RETINAL AXON CROSSING AT THE ZEBRAFISH OPTIC CHIASM

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During development, axons navigate a complex intracellular milieu, often over long distances to reach target tissues at stereotyped locations. The basis for this navigation, lies in the growth cone – a motile structure at the tip of the growing axon. Studded with receptors for an array of guidance cues, the growth cone interprets a balance of attractive and repellant signals en route to its target. However, during pathfinding, axons often must extend along or near areas expressing repellents. We previously showed that growth cones can modulate their response to multiple repellents through a G protein coupled receptor, calmodulin, cAMP and PKA mediated pathway that ultimately abrogates the repellent response through inhibition of Rho. (Chalasani et al, 2003 PMID:12598624) We sought to further investigate the role of cAMP and G Protein signaling in guidance – and used the zebrafish retinto-tectal projection as our model system, due to its well characterized projection – all axons extend towards the ventral midline, cross at chiasm and project dorsally and posteriorly to the contralateral tectum. In Chapter 2, I describe my contributions to a paper recently published (Xu, et al 2010 PMID:20505109), in which we describe the role of the calcium/calmodulin adenylyl cyclase, ADCY8, in directing the trajectory of retinal ganglion cells. In Chapter 3 I describe a study that examined the role of G protein coupled receptor signaling in directing retinal trajectories. In this study, I took advantage of the UAS/GAL4 system to express dominant negative constructs targeting specific classes of heterotrimeric G

proteins in retinal ganglion cells. We generated constructs targeting Gαi/o, Gαq/11, Gαs/olf, and Gβγ. We find that reduction of GαS produces a consistent guidance error – misprojection to the ipsilateral tectum. These errors phenocopy the knockdown of ADCY8 as well as knockdown of the axon guidance molecules Semaphorin 3D, Semaphorin 3E and their co-receptor, Nrp1a. Using a combination of QPCR, morpholino injection, and in vivo manipulation we demonstrate that depression of cAMP signaling, either through the heterotrimeric G protein GαS or ADCY8 reduces Neuropilin expression, and renders navigating axons insensitive to Semaphorin 3D and Semaphorin 3E signaling near the chiasm.

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CHAPTER 1: GENERAL INTRODUCTION

Molecular Mechanisms of Axon Guidance

In order to generate a functional neural circuit, individual neurons must forge connections with their appropriate synaptic partners. Once a newly born neuron has assumed a particular neuronal identity and migrated to its appropriate location, it sends out a long axonal process, navigating successive local intermediate targets over long distances in order to achieve appropriate connectivity. Dissecting the signaling pathways and cytoskeletal mechanisms that underlie growth cone dynamics and axonal pathfinding has been the focus of intense study over the last three decades. In the following pages I will focus on the identification of extracellular guidance cues that direct growing axons, and how these cues mediate a fundamental choice many axons must make – weather or not to decussate, or cross the midline. Classically, guidance cues have been categorized by their ability to attract or repel axonal growth cones in culture. However, the way a given guidance cue is interpreted depends on both surface expression of the appropriate receptor(s) as well as intracellular state of the navigating growth cone. Recently, the field has begun to interrogate how growing axons integrate multiple cues, concurrent with the complex extracellular milieu they encounter along their journey to their targets.

Technical Approaches

Several *in vitro* assays have been devised to assess axonal response to candidate guidance cues, and parse the signaling pathways associated with these cues. In a classic turning assay, a defined concentration of a candidate cue is applied at a distance from cultured growth cones and allowed to diffuse through the culture medium (Lohof et al., 1992; Pujic et al., 2008). This assay has recently been adapted to better monitor the

stop-and-start growth patterns of mammalian commissural neurons using the Dunn chamber (Yam et al., 2009). In coculture assays, neuronal explants are cultured at a distance from transfected cells expressing a candidate cue (Serafini et al., 1996). If the cue is unknown, a candidate cue-secreting tissue may be cocultured at a distance from growing axons (Tessier-Lavigne et al., 1988; Placzek et al., 1990).

Collapse assays, carried out on cultured axons, serve to quantify the strength of repellents by monitoring the withdrawal of growth cone lamellae and filopodia (collapse) in the presence of varying concentrations of repellent cue (Raper and Kapfhammer, 1990; Kapfhammer et al., 2007). Membrane bound cues can be assessed using the stripe assay – in which either membrane fractions or purified candidate cues are applied in a defined pattern to a culture plate, and the axonal outgrowth on, or off the stripes defines the role of the guidance cue to that population of neurons (Knöll et al., 2007).

In the mid 1980s monoclonal antibody (mAb) screens in Drosophila followed by biochemical analysis of the antigen identified cell surface proteins that defined particular axonal tracts (Bastiani et al., 1987; Patel et al., 1987; Kolodkin et al., 1992). These screens complemented similar antibody screens carried out in the rat and chick spinal cord (Jessell et al., 1990; Stoeckli and Landmesser, 1995), and in the Xenopus retino-tectal system. (Takagi et al., 1987) Genetic screens followed as natural extension of these antibody screens (as the antibodies provided a way to label specific axonal projections) and led to the identification of mutants in which these projections, or other identifiable axonal tracts were perturbed. For example, Seeger et al (1993) used the screen-identified mAb, BP102, which labels the anterior and posterior commissures and longitudinal axonal tracts in the Drosophila ventral nerve cord, to identify 250 mutants with disrupted axonal tracts, including *comm* and *robo*.

Guidance cues: Slits, Semaphorins, Ephs/Ephrins and Netrins

The combined approach of *in vitro* biochemical manipulation and genetic and biochemical screens led to identification of four families of canonical axon guidance cues: Slits, Semaphorins, Ephs/Ephrins, and Netrins, which act at a distance or upon cell-cell contact. (Tessier-Lavigne and Goodman, 1996; Huber et al., 2003) In addition to these, the morphogens Sonic Hedgehog (Shh), Bone Morphogenic Proteins (BMPs) and Wnts can direct the trajectories of commissural axons independent of their ability to confer neuronal identity. (Augsburger et al., 1999; Charron et al., 2003; Lyuksyutova et al., 2003) Cell adhesion molecules such as L1 and NrCAM complex with canonical signaling pathways, or act independently to promote guidance and fasciculation in growing neurons. (Stoeckli et al., 1997; Lustig et al., 1999; Castellani et al., 2000)

Guidance cues at work: Midline Crossing

Models of midline crossing, including the Drosophila ventral nerve cord, the commissural axons of the vertebrate spinal cord, and vertebrate retino-tectal projection have proved particularly useful in parsing related dilemmas underlying the establishment of neural circuits: whether, where, and how to cross the midline. Once at an attractive intermediate choice point, how do growth cones move on to navigate towards their final synaptic targets? Within the Drosophila ventral nerve cord and the vertebrate spinal cord, the initial extension of axons to their targets is mediated both by attraction and by blocking the navigating axons' perception of the repulsive cues that will direct them away from the midline after crossing (reviewed in Dickson and Zou, 2010) This "repellent deafness" can occur either by limiting the surface expression of a repellent receptor, or by local modulation of cyclic nucleotides such as cAMP within the navigating growth cone – either way, the process must be tightly controlled in both time and space (Chalasani et al., 2003; Keleman et al., 2005; Parra and Zou, 2010; Charoy et al., 2012).

In Chapter 3 of this document I show that cAMP signaling downstream of G protein coupled receptors (GPCRs) and the Ca^{2+}/Calmodulin adenylyl cylcase ADCY8, promotes the expression of a key component of Semaphorin signaling – the co-receptor Nrp1a. RGCs require Nrp1a in order to interpret signals from ligands Sema3D and 3E at the chiasm. I also contributed to a study that examined the role of ADCY8 in RGC crossing which identified a role for cAMP in antagonizing Slit/Robo signaling at the chaism, which is presented in Chapter 2.

At midline choice points such as the *Drosophila* ventral nerve cord, the spinal cord floor plate, and the optic chiasm, navigating growth cones are presented with multiple cues, both attractant and repellent, which must be appropriately integrated to produce an accurate pathfinding decision. Here I highlight the conserved functions of netrin and Slit/Robo signaling in midline axon guidance, in the context of core questions that still remain largely unanswered in the retino-tectal system -- getting axons to, and away from the midline. Semaphorin/Plexin signaling is addressed separately at the end of this section.

Vertebrate Spinal Cord: Netrin is a conserved guidance cue that attracts axons to the midline.

Cell bodies of vertebrate commissural axons reside in the dorsal spinal cord, and send axons ventrally towards, and across the glial cells at the ventral midline (floorplate) where they subsequently project anteriorly towards brainstem targets. Precrossing commissural axons express the axonal glycoprotein TAG1, while post crossing axons express L1 (Dodd et al., 1988). Co-culture experiments revealed that floorplate explants attracted TAG1 + commissural axons (Tessier-Lavigne et al., 1988; Placzek et al., 1990). Screening embryonic brain extracts for factors which could promote outgrowth of

commissural axons led to the identification of two laminin-like proteins, Netrins 1, and 2 – with homology to *c.elegans* unc-6 (Hedgecock et al., 1990; Kennedy et al., 1994). Though Netrins 1 and 2 are both expressed in the ventral spinal cord, and both are able to reorient pre-crossing commissural axons in vitro, only Netrin 1 is expressed in the floor plate (Kennedy et al., 1994). Netrin transduces attractive cues via the related DCC and neogenin receptors, or DSCAM (Fazeli et al., 1997; Meyerhardt et al., 1997; Ly et al., 2008). In mouse Netrin $1^{-/-}$ mutants, many axons stall midway down the spinal cord or fail to cross the midline (Serafini et al., 1996). In *Drosophila*, NetrinA/B or Frazzled (DCC) mutants also display thin or absent commissures, though, surprisingly, Netrin need not be secreted in order to direct the orientation of Drosophila commissural axons (Harris et al., 1996; Kolodziej et al., 1996; Brankatschk and Dickson, 2006).

Some commissural axons still cross the floor plate in Netrin mutants, suggesting the presence of another attractant or permissive factor in this tissue. The cue was eventually identified as the morphogen Sonic Hedgehog (Shh), acting through a non-canonical Smoothened and Src family kinase (SFK) dependent mechanism (Charron et al., 2003; Yam et al., 2009). Thus, attraction by netrin is a conserved guidance mechanism that promotes axons getting to an intermediate target.

Drosophila Ventral Nerve Cord: Slit/Robo signaling promotes exit from the midline and prevents recrossing. Comm prevents premature sensitivity to Slit.

In the Drosophila ventral nerve cord (VNC), motor neurons, commissural neurons, and ipsilateral (longitudinal) neurons comingle. The VNC, labeled by BP102 antibody staining resembles a segmented ladder - regular array of commissures, flanked by the "rails" of the ladder, the longitudinal axons, three fascicles of which are labeled by the Fas II (ID4) antibody. Mutagenesis screens in Drosophila identified mutants in which

either too many axons cross (*slit/robo*), or no axons cross (*comm*) the ventral midline. (Seeger et al., 1993)

In *slit* mutants, all axons, longitudinal or commissural, project to the midline and stay there. The *slit* mutation was originally identified in a large-scale screen for cuticle defects, and was independently discovered in a library screen for EGF like proteins (Rothberg et al., 1988). The BP102 axon guidance screen also identified 13 alleles of Slit (Seeger et al., 1993, cited in Kidd et al., 1999). Slit mRNA and protein is expressed in midline glial cells analogous to the floorplate, and encodes a 190kd full length protein with multiple EGF and leucine rich repeats (Rothberg et al., 1988; Kidd et al., 1999). Slit family members contain a conserved proteolytic domain between EGF repeats 5 and 6 (Brose et al., 1999; Wang et al., 1999). However, cleavage of Slit doesn't seem to be required for guidance, as an uncleavable Slit rescues the axon defects in the *slit* mutant (Coleman et al., 2010). In mouse, Slits1,2, and 3 are expressed in the floorplate, while Slit's receptor, Robo, is expressed in commissural axons (Kidd et al., 1998; Brose et al., 1999). *Slit1*$^{-/-}$*;Slit2*$^{-/-}$*;Slit3*$^{-/-}$ mouse mutant commissural axons stall and defasiculate at the floor plate. Mutant commissural axons also recross the floor plate, similar to the *Drosophila robo* mutant (Long et al., 2004).

Turning down repulsion

Robo mRNA is broadly expressed in the CNS, but wholemount and immuno-electron histochemistry using an antibody directed at the Robo's extracellular domain revealed that the protein is restricted to the surface of non-crossing longitudinal axons, or on post-crossing commissural axons, but is also present in vesicles within crossing axons. (Kidd et al., 1998) Since Robo is expressed on the surface of axons only after they reach the

midline, its expression must be tightly controlled. In contrast to *slit* and *robo*, *comm* mutant axons remain longitudinal – no axons cross the midline, phenocopying Robo overexpression (Kidd et al., 1999). Comm is not expressed on longitudinal axons and is required cell autonomously in commissural axons for crossing (Georgiou and Tear, 2002; Keleman et al., 2002, 2005). Comm/Robo double mutants display a Robo-like phenotype suggesting that Comm usually functions to repress Robo in crossing axons (Kidd et al., 1998). In order to examine the trafficking of Robo and Comm, Keleman et al (2005) expressed a Robo:GFP fusion protein in Comm negative peripheral axons, with or without Comm overexpression. In the absence of Comm, the fusion protein was localized at the surface of the axon, as well as in fast moving antero- and retrograde puncta within the cytoplasm. Upon Comm expression, Robo:GFP expression decreases at the plasma membrane and increases in slow moving cytoplasmic puncta in the cell soma and axon (Keleman et al., 2005). The model that emerged is that Comm sorts Robo to endosomes to keep it from the midline until after the axons have crossed. Surprisingly, a sorting defective Robo, RoboSD, which cannot bind Comm, displays an axonal scaffold indistinguishable from WT, suggesting a role for Comm independent of its role in sequestering Robo (Gilestro, 2008). After crossing, Comm expression is reduced, Robo is trafficked to the surface and axons are repelled from the midline in response to Slit. In the vertebrate spinal cord, the 3.1 splice isoform of the divergent Robo receptor Rig1/Robo3 is expressed on precrossing axons, and functions analogously to comm to reduce the sensitivity of precrossing axons to slit (Sabatier et al., 2004; Chen et al., 2008). Robo3-/- (Rig1) commissural axons stall before entering the floorplate and fail to cross the midline, consistent with a premature sensitivity to slits observed in explant culture (Sabatier et al., 2004).

Slit/Robo and Netrin signaling pathways interact to fine-tune expression and function of guidance receptors at the midline, during crossing. For example, the expression of a dominant negative form of the Netrin receptor Fra produces a Comm like phenotype and Comm expression levels are reduced in Fra mutants independent of netrin (Yang et al., 2009). *In vitro* binding assays suggest that Netrin can bind Slit via its LRR domains, and it has been proposed that Slit signaling can silence Netrin mediated attraction by interaction of their receptors' (Robo and DCC respectively) cytoplasmic domains (Brose et al., 1999; Stein and Tessier-Lavigne, 2001).

Vertebrate Optic Chiasm: Slit/Robo signaling accounts for where retinal axons cross, but not how they cross. Semaphorin signaling facilitates midline crossing.

The neural architecture of the retina has been extensively studied, and the information flow within the retina is conserved between insects and vertebrates (Sanes and Zipursky 2010). Retinal Ganglion Cells (RGCs), the projection neurons of the retina, must export spatial information to higher brain centers, and thus project topographically to their targets, crossing the midline on the way. The projection of RGC axons is an excellent model to study several aspects of axon guidance, including midline crossing, topographic mapping, and arborization.

RGCs are a heterogeneous mix of cells, characterized into 22 types based on their morphologies (Völgyi et al., 2009). Following differentiation, RGCs extend axons towards the optic nerve head, and exit into the optic tract, where they cross at the ventral midline before projecting to their synaptic targets. RGC growth cones are labile structures – speedy, elongated and bullet-like along the optic tract, and paused, elaborated into complex structures at navigational choice points, such as the midline (Bovolenta and Mason, 1987). In humans and mouse, some RGCs do not cross the

midline, projecting to ipsilateral targets, while others project contralaterally across the midline. In animals lacking binocular vision, such as zebrafish, all RGC axons project contralaterally (Rasband et al., 2003). This all-contralateral projection, together with its optical transparency, and genetic tractability makes the zebrafish an excellent model to study midline crossing. Forward genetic screens in zebrafish identified mutants defective in axonal sorting in the tract, crossing and the midline, and lamination, topographic targeting, and arborization in the tectum - which have complimented existing studies in mouse and chick (Baier et al., 1996; Karlstrom et al., 1996; Trowe et al., 1996; Xiao et al., 2005).

Axon Guidance at the Chiasm

Perhaps due to their distinct lineage, retinal midline crossing differs in several respects from the commissural axons of the spinal cord (Williams et al., 2004). In mouse, Netrin is not expressed at the chiasm, though it does attract axons to the eye exit point (Deiner et al., 1997). In the absence of an identified midline attractant, several lines of evidence suggest that Slit/Robo signaling dictates where RGC axons will cross the midline. In mouse, chick and zebrafish, RGC growth cones express the receptor Robo2 for repellents Slit1, Slit2, and Slit3, which line the path axons must take (Erskine et al., 2000; Niclou et al., 2000; Hutson et al., 2003). *Astray* (Robo2) mutant zebrafish, and mouse Slit1/2 double mutants each display severe axonal guidance errors, including the formation of ectopic commisures, as well as the formation of ipsilateral misprojections, and sprouting into the contralateral optic tract (Baier et al., 1996; Fricke et al., 2001; Plump et al., 2002). These ectopic anterior “chiasms” suggest that Slits control where axons should cross the midline, but not how they cross. Many *astray* and *slit1/2* mutant axons do make it to their targets, raising the possibility that additional factors are

required to promote or prevent RGC midline crossing. In higher vertebrates, a subset of retinal axons project ipsilaterally. In the mouse, about 4% of axons, which arise in the VT retina, express the transcription factor Zic1, which promotes expression of the membrane Ephb1, deflecting these axons away from the midline (Herrera et al., 2003). Intriguingly, some later born axons from the VT retina do project contralaterally suggesting an independent program promotes crossing. Recently another family of canonical guidance cues, Semaphorins, along with receptors, Neuropilins and Plexins have been identified in a conserved program to direct crossing of contralaterally projecting RGC axons (Sakai and Halloran, 2006; Erskine et al., 2011; Kuwajima et al., 2012). In Chapter 3, I describe a mechanism by which cAMP promotes Neuropilin expression in retinal ganglion cells, enabling them to respond to Semaphorins near the midline.

Semaphorins: Axonal Glycoproteins that attract or repel axons

The Semaphorin family of axonal glycoproteins encompass one set of canonical guidance cues. These molecules were first identified by their repellent activity on growing axons. Luo et al isolated a 100kd protein from chick brain that induced DRG axons to stall and collapse (Luo et al., 1993). This protein, Collapsin-1/Sema3A, contained a domain with a homology to grasshopper FasIV, now known as Sema1 (Kolodkin et al., 1992, 1993; Luo et al., 1993). Recent experiments using optical tweezers to deliver defined amounts of Sema3A to cultured hippocampal neurons suggest that as few as 200 molecules of Sema3A are sufficient to induce repulsion (Pinato et al., 2012).

The Semaphorins (Semas) are now known to comprise a large, (20+) family of secreted, transmembrane, or viral glycoproteins, identified by their PSI (Plexins-Semaphorins-

Integrins) and eponymous 500aa Sema domains. Classes 1 and 2 comprise invertebrate semaphorins. In vertebrates, Class 3 semaphorins are secreted, while classes 4-7 are membrane bound. The eighth class constitutes viral semaphorins. (Raper, 2000; Pasterkamp and Giger, 2009) Both secreted and membrane bound Semaphorins can direct axonal growth. For example, the secreted Semaphorin Sema3B is required for formation of the anterior commissure. (Falk et al., 2005) Membrane bound Sema6D can direct the trajectories of both sensory and motor axons, and as well as retinal ganglion cell axons. (Yoshida et al., 2006; Kuwajima et al., 2012)

Sema3A has served as a well-studied model for class 3 semaphorins. In addition to the Sema and PSI domains that characterize the family, class 3 Semas also include Ig loops, as well as a c-terminal basic domain unique to the secreted semaphorins. A 70aa stretch within the Sema domain is required for repellent activity among the various class 3 Semas, as swapping the 70aa stretch from Sema3A into Sema3D, produces a collapse profile similar to Sema3A and vice versa. (Koppel et al., 1997) Sema3A's repellent activity is potentiated by its homodimerization via disulfide bonds, as non-dimerizing mutants (C723A) display a 40-fold reduction in their ability to collapse DRG growth cones. (Koppel and Raper, 1998) Semaphorin3A and 4D crystal structures have been solved, revealing the Sema domain as a variant of the beta-propeller structure which also characterizes integrins (Antipenko et al., 2003; Love et al., 2003; Janssen et al., 2010)

Semaphorin Receptor Complexes

Class 3 semaphorins signal through a holoreceptor complex that includes Neuropilins and Plexins, as well as CAMs like L1 or NRCAM.

Neuropilins

Neuropilin1 was first identified and characterized via a monoclonal antibody screen targeting the Xenopus optic tectum. One such antibody, mAb5 recognized a 140kd protein, the A5 antigen. The protein was further characterized by screening the antisera against a library of Xenopus cDNAs and reconstructing the sequence from 12 partially overlapping sequences, eventually identifying a 3kb DNA sequence with a hydoprathy profile suggesting a single pass transmembrane protein (Takagi et al., 1987, 1991). The protein, which they later named neuropilin, is conserved in chick and mouse, and axonally localized (Kawakami et al., 1996).

Two independent studies identified Neuropilin 1 as a receptor for class three Semas. Both studies used an alkaline phosphatase tagged Sema 3 (AP-Sema3) to screen cDNA expression library generated from embryonic DRGs, or a mixed library from DRG and spinal cord (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). CoIP and structure function experiments demonstrated that the proteins directly interact and that the C and Sema domains of Sema3 mediate neuropilin binding. (He and Tessier Lavigne, 1997). Anti-Neuropilin antibody inhibited the repellent response of DRG axons to sema3A (He and Tessier Lavigne. 1997, Kolodkin et al., 1997). The study from Kolodkin and colleagues also identified and cloned Neuropilin-2 based on ESTs with similarity to Nrp1. Nrp2 was independently identified using degenerate PCR targeting conserved Nrp domains. (Chen et al., 1997; Kolodkin et al., 1997) Npn-1 features an N-terminal (CUB) domain, a coagulation factor V/VIII homology domain, MAM domain, as well as short transmembrane (23aa) and cytoplasmic (39aa) regions (Kawakami et al., 1996). Though domain organization is conserved between Nrp1 and Nrp2, the protein sequences are different, and they are expressed on partially overlapping subsets of neurons (Chen et

al., 1997; Kolodkin et al., 1997). Chick RGCs expressing Nrp1 deletion constructs lacking Nrp1's CUB or MAM domains do not collapse in response to Sema3A, while full length and cytoplasmic deletions collapse robustly, leading to the conclusion that Nrp's primary function is to capture Sema and present it to co-receptor Plexin (Nakamura et al., 1998). Recent studies in Nrp1's alternate role as a VEGF co-receptor during angiogenesis, suggest that three conserved amino acids in the c-terminal might function to recruit RGS-GaiP, a negative regulator of the heterotrimeric G protein Gαi, though it remains to be seen if this happens in axons (Wang et al., 2006).

Plexins

Plexins are canonical transducers of Sema signaling activity. Class 3 semaphorins 3A-D, F and G bind PlexinsA1-4 via neuropilin, while membrane bound Semaphorins do not require neuropilin for signaling. (Reviewed in Pasterkamp and Giger, 2009) Like Neuropilin, Plexin was first identified through a monoclonal antibody screen directed towards the *Xenopus* optic tectum, where it was recognized by the B2 mAB (Takagi et al., 1987; Ohta et al., 1995). Plexin's extracellular domains feature cysteine rich domains homologous to Met like Scatter factor receptors, as well a Sema domain. (Winberg et al., 1998; Tamagnone et al., 1999; Rohm et al., 2000) Plexins coIP with Neuropilins and both PlexinB1 and PlexinA3 are tyrosine phosphorylated, consistent with conserved cytoplasmic Y phosphorylation motifs. (Tamanone et al., 1999) PlexinA mutants phenocopy the motor axon guidance defect observed in SemaA mutants (Winberg et al., 1998). Additionally, truncated Plexin acts as a dominant negative to abolish turning response to Semaphorin3a in Xenopus spinal neurons, chick DRG and mouse sensory axons (Takahashi et al., 1999; Tamagnone et al., 1999; Rohm et al., 2000).

IgCAMs

IgCAMs have also been implicated as components of Semaphorin/Plexin signaling. WT cortical axons typically orient away from sources of Sema3A. However, cortical axons lacking the IgCAM L1 are not repelled by sources of Sema3A (Castellani et al., 2000; Law et al., 2008). L1 coIPs with Nrp1 in overexpression experiments in cos cells, and a soluble L1-Fc blocks Sema3A mediated growth cone collapse on cultured neurons (Castellani et al., 2000). Surface dextran labeling experiments in cortical axons suggests that L1 might act to potentiate Sema3A signaling by contributing to endocytosis of Nrp1a, as L1 and Nrp1A are co-endocytosed upon Sema3A stimulation. TRKA+ DRG axons in TAG1 or L1 mutant mice prematurely project into the dorsal horn, as a result of insensitivity to Sema3A, mediated by a disruption of TAG1/L1 interactions, and correlating with a reduction in endocytosis (Law et al., 2008). Here TAG1 seems to act intracellularly to ensure that neuropilin is trafficked away from endosomal L1 to lipid rafts (Dang et al 2012).

Attractant and Repellent signaling through Semaphorins

Semaphorins can mediate attractive or repellent responses in growing axons. Class 3 semaphorins were initially characterized as repellents. Sema3A collapses DRG axons, and DRG axons of Sema3A or Nrp1 mouse knockouts aberrantly extend into the ventral spinal cord (Luo et al., 1993; Behar et al., 1996). Sema3A also repels and inhibits branching of cortical axons (Bagnard et al., 1998; Dent et al., 2004). Sema3C attracts colossal axons via Nrp1, and is attractive to cortical axons based on stripe assays, but acts as a repellent for sympathetic axons (Bagnard et al., 1998; Chen et al., 1998; Niquille et al., 2009). Sema3D and 3E, which I will discuss in greater detail in Chapters

3 and 4, have each been reported to act as both attractants and repellents. Consistent with Sema3D acting as an attractant, Sema3D overexpression causes habenular neurons to overshoot their targets (Kuan et al., 2007). Zebrafish anterior commissure axons extend towards mosaic patches of Sema3D (Wolman et al., 2004). However, Sema3D has also been reported to be a repellent for RGCs in the optic tectum, and repels peripheral sensory axon branches (Liu et al., 2004; Liu and Halloran, 2005). Sema3E is unique because it can bind its cognate plexin independently of Nrp1 (Gu et al., 2005). Sema3E acts as an attractant for subiculomammilary axons through an interaction that includes Nrp1, but repels corticofugal axons via PlexinD1 independent of Nrp1 (Chauvet et al., 2007). We have also previously shown that cyclic nucleotide signaling through GPCRs can decrease the repellent effects of Sema3A in chick DRG growth cones.

Cyclic nucleotides influence growth cone response to guidance cues.

One canonical way to switch attractive and repulsive growth cone responses is by manipulating the concentration of cyclic nucleotides within the growth cone. cAMP, cGMP or Ca^{2+} can mediate growth cone responses locally, over short time scales. (Song et al., 1998; Gomez and Zheng, 2006) A series of studies based on turning assays in Xenopus spinal neurons explored the role of signaling via second messengers Ca^{2+} and cyclic nucleotides cGMP and cAMP in growing axons. (Ming et al., 1997; Song et al., 1997, 1998; Moore and Kennedy, 2006) These studies revealed that cyclic nucleotide signaling can mediate both modulatory and instructive cues for growth cones. Spinal axons turn away from a source of Sema3A, but that response is switched to attraction in the presence of cGMP agonists. Though cAMP agonists had no effect on Sema mediated repulsion by themselves, they blocked cGMPs switching effect (Song et al.,

1998). In addition to mediating attractive signaling via DCC, Netrin also transduces repellent signaling through receptor Unc-5. The attractive signal is switched to repulsion in the presence of cAMP or PKA inhibitors. (Ming et al., 1997; Nishiyama et al., 2003) In contrast, repellent Unc5 mediated repulsion is blocked by inhibition of PKA, and converted to attraction in the presence of PKG antagonist KT5720. By systematically varying the ratio of cAMP/cGMP but keeping the total concentration the same, Movshon and colleagues demonstrated that a high ratio of cAMP/cGMP mediates growth cone attraction, whilst a low ratio results in repulsion (Nishiyama et al., 2003).

We have demonstrated that cAMP signaling via GPCRs can reduce axonal response to repellents in vitro. In zebrafish and chick retinal ganglion cells, application of SDF reduces growth cone responses to the repellent Slit2, concurrent with a calmodulin dependent increase in cAMP (Chalasani et al., 2003, 2007; Xu et al., 2010). Over short time scales, cAMP can antagonize Semaphorin/Plexin signaling. The Drosophila Nervy mutant encodes an AKAP, which directly associates with Plexin, and antagonizes Sema signaling via PKA (Terman and Kolodkin, 2004). In explants of mouse commissural axons, Shh potentiates the repellent responses to Sema3B and 3F, via a mechanism that is blocked by application of forskolin (Parra and Zou, 2010). cAMP also can impact axon guidance by perturbing gene expression, via PKA's phosphorylation of CREB and other bZIP transcription factors. CREB mutant mice display severe axon guidance perturbations, including defects in midline crossing as the anterior commissure fails to form. (Rudolph et al., 1998; Lonze et al., 2002) cAMP can also impact gene expression and axon guidance in a PKA independent manner, via EPAC (Murray and Shewan, 2008; Sands and Palmer, 2008; Peace and Shewan, 2011). In the studies presented in Chapter 2, we demonstrate that cAMP acts in a calmodulin dependent manner, over

short time-scales to reduce RGC axons response to Robo and help axons across the midline (Xu et al., 2010). In Chapter 3, we show that ADCYs and heterotrimeric G proteins are required to maintain cAMP levels and contribute to axon guidance by regulating Neuropilin gene expression.

Adenylyl cyclases produce cAMP and are regulated by heterotrimeric G proteins.

cAMP levels are regulated by adenylyl cyclases (ADCYs), which produce cAMP from ATP upon catalytic binding to the C1 or C2 domains of one of the nine membrane bound adenylyl cyclases. (Sadana and Dessauer, 2009). ADCYs are grouped into families based on their modes of regulation, which largely occurs via heterotrimeric G proteins signaling downstream of GPCRs. *Group 1 ADCYs*, including ADCY1, 3, and 8 are stimulated by Ca^{2+} /Calmodulin. *Group 2 ADCYs* 2, 4 and 7 are stimulated by G®©. *Group 3 ADCYs* 5, 6 are inhibited by Gαi and Ca^{2+}. In *Group 4* ADCY9 is forskolin insensitive.

Heterotrimeric G proteins are critical effectors ADCY regulation. GαS can activate all ADCYs in vitro. Gαi inhibits calmodulin stimulated AC1, as well as GαS stimulated ADCYs 5,6; however, other ADCYs are insensitive to Gαi. (Taussig et al., 1994; Sadana and Dessauer, 2009) ADCYs can also be activated by PKCα. (Kawabe et al., 1994) The calmodulin stimulated ADCYs ADCY1 and 8 are highly expressed in the brain; and I contributed to a recent publication from our lab demonstrating that ADCYs are required for retinal axons to cross the midline in the developing zebrafish, which is included here as chapter 2 (Antoni et al., 1998; Nicol et al., 2005; Xu et al., 2010).

G protein coupled receptor signaling through heterotrimeric G proteins can directly and locally impact the status of cyclic nucleotide signaling at the cell membrane by stimulating adenylyl cyclases, as well as couple to other pathways that result in cytoskeletal changes. G-protein coupled receptors converge on a limited number of effectors, the heterotrimeric G proteins. Humans have 21 α subunits (16 genes) and 6 β (5 genes) and 5 γ subunits (Downes and Gautam, 1999; Oldham and Hamm, 2008). Heterotrimers fall into 4 families, based on the taxonomy of the alpha subunit: GαS, Gαi, GαQ and Gα12/13. As discussed above, signaling through GαS potentiates cAMP signaling, while Gαi inhibits select ADCYs. However, Gαi/o families can also potentiate the GTPases Rac and Rho, and signal via PI3K. GαQ is canonically coupled to PKC and Calmodulin activation, while Gα12/13 can directly activate small G proteins such as cdc42 and Rho. (Cotton and Claing, 2009) Interestingly GαO can localize to axons via a direct interaction with the membrane protein GAP43 (Strittmatter et al., 1990). Concurrent with their broad expression patterns, mouse knockouts for the various alpha subunits are often embryonic lethal. Gαi, GαO, Gαolf, GαQ and GαS mutants all display neurological defects including hyperactivity, tremors, seizures, and ataxia (Offermanns et al., 1997; Valenzuela et al., 1997; Jiang et al., 1998; Huang et al., 2006).

Thus heterotrimeric G protein function is required for the appropriate establishment and function of neural circuits, but knockouts display pleiotropic effects that impede analysis of G protein impact on axon guidance. I developed a set of dominant negative reagents that interfere with specific classes of heterotrimeric G proteins, and can be expressed in individual subsets of neurons using the UAS/Gal4 system. I took advantage of the stereotyped projection of the zebrafish retino tectal system used these reagents in the

set of studies to interrogate the role of G protein signaling in midline crossing. The results of these studies are described in Chapter

CHAPTER 2: THE CALMODULIN STIMULATED ADENYLATE CYCLASE ADCY8 SETS THE SENSITIVITY OF ZEBRAFISH RETINAL AXONS TO MIDLINE REPELLENTS AND IS REQUIRED FOR NORMAL MIDLINE CROSSING.

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During my second and third years, I collaborated with postdoctoral fellow Hong Xu on a project to examine the role of Ca^{2+}/calmodulin mediated adenylate cyclases ADCY1 and ADCY8 in the zebrafish retino-tectal projection. I carried out numerous in situ hybridizations to demonstrate that ADCY8 is expressed in retinal ganglion cells, and that expression of Slits, Semaphorins, and Sonic Hedgehog are unchanged at the midline of ADCY8 morphants.

ABSTRACT

The chemokine SDF1 activates a cAMP mediated signaling pathway which antagonizes retinal responses to the midline repellent slit. Using the zebrafish (*Danio rerio*), we show that knocking down the calmodulin activated adenylate cyclase ADCY8 makes retinal axons insensitive to the SDF1. Experiments *in vivo* confirm a mutual antagonism between slit signaling and ADCY8 mediated signaling. Unexpectedly, knockdown of ADCY8 or another calmodulin activated cyclase, ADCY1, induces ipsilateral mis-projections of retinal axons that would normally cross the ventral midline. We demonstrate a cell autonomous requirement for ADCY8 in retinal neurons for normal midline crossing. These findings are the first to show that ADCY8 is required for axonal pathfinding before axons reach their targets. They support a model in which ADCY8 is an essential component of a signaling pathway that opposes repellent signaling. Finally, they demonstrate that ADCY8 helps regulate retinal sensitivity to midline guidance cues.

INTRODUCTION

Axons navigate through the developing nervous system by responding to differentially localized guidance cues in their immediate environment. Many diverse families of secreted and cell surface signaling molecules have been found to guide axons through their attractive and repellent effects on growth cones (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). Growth cones routinely encounter multiple cues simultaneously and must integrate their activities into unitary guidance decisions. Understanding how axons navigate through their native environment will require an understanding of how multiple cues interact with one another to affect growth cone behavior.

One such interaction is the antagonistic relationship between the chemokine SDF1 and repellent guidance cues. SDF1 has been shown to make a variety of axons less sensitive to multiple axonal repellents including semaphorin3A, semaphorin3C, and slit2 *in vitro* (Chalasani et al., 2003). It has also been shown to antagonize slit/robo signaling *in vivo* (Chalasani et al., 2007). Pharmacological studies in cultured chick sensory and retinal axons showed that SDF1's anti-repellent effect is mediated by the seven transmembrane receptor CXCR4, a pertusis toxin-sensitive G-protein coupled pathway, the stimulation of calcium-calmodulin, and the activation of PKA. An analogue of cAMP stimulates the anti-repellent pathway while a cAMP antagonist blocks the anti-repellent effects of SDF1 (Chalasani et al., 2003). These results suggest that SDF1 signals through a calmodulin activated intermediary to elevate cAMP. Altering levels of cyclic nucleotides has been shown to dramatically alter growth cone responses to both attractants and repellents *in vitro* (Song and Poo, 1999; Piper et al., 2007), but there is

very little understanding of how these levels are controlled *in vivo*. There are ten recognized adenylate cyclases that produce cAMP in higher vertebrates (Willoughby and Cooper, 2007). Two of them, ADCY1 and ADCY8, are known to be activated when they bind calcium activated calmodulin (Ferguson and Storm, 2004). ADCY1 is maximally activated by a combination of activated GαS and calmodulin, while ADCY8 is activated by calmodulin alone (Wayman et al., 1994; Nielsen et al., 1996). Since SDF1 initiates its anti-repellent effects through a pertussis toxin sensitive Ga_i or Ga_o, and since it is not blocked by interfering with GαS-mediated signaling (Twery et. al., unpublished), we hypothesized that SDF1's anti-repellent effects are mediated by calmodulin induced ADCY8 generation of cAMP. We therefore tested whether knockdown of ADCY8 interferes with SDF1-mediated anti-repellent signaling in cultured zebrafish retinal axons and during retinal pathfinding in the embryonic zebrafish.

Consistent with the hypothesis that calmodulin-activated cyclases participate in the SDF1 activated anti-repellent pathway, knockdown of ADCY8 makes zebrafish retinal axons more responsive to slits in the presence of SDF1 *in vitro*. Either ADCY8 or SDF1 knockdown tends to rescue specific retinal misprojections in mutant embryos in which retinal sensitivity to midline repellents is reduced. Surprisingly, knockdown of zADCY8 in the embryo induces abnormal ipsilateral retinal projections, and these mis-projections can be rescued by the simultaneous reduction or elimination of slit/robo repellent signaling. These findings demonstrate a requirement for the calmodulin-activated adenylate cyclase ADCY8 in retinal axon pathfinding *in vivo* and they are consistent with a model in which ADCY8 is part of a pathway that antagonizes repellent slits expressed at the ventral midline.

MATERIALS AND METHODS

FRET imaging. Changes in cAMP levels were monitored in retinal ganglion cells by Fluorescence Resonance Energy Transfer (FRET) using the cAMP sensor ICUE3 (DiPilato and Zhang, 2009). E5 chicken retinae were dissected and dissociated after treatement with 0.25% trypsin for 12 min at 37℃. Dissociated retinal neurons were electroporated with ICUE3 plasmid using the rat neuron Nucleofector kit and plated onto the glass bottoms of MatTak dish coated with laminin. Neurons were allowed to attach after plating for 6 hours in F12 medium supplemented with 6 mg/ml glucose, 2mM glutamine, 100 μM putrescine, 200 μM progesterone, 5 μg/ml insulin, 20 μg/ml NGF, 100 U/ml penicillin, 100 μg/ml streptomycin and 500 μl bovine pituitary extract (BPE) per 100 ml. The medium was then exchanged with F12 medium supplemented with all of the above components except BPE and cultured overnight at 37℃ and 5% CO_2. On the second day, the F12 medium was replaced with L15 medium supplemented with all of the above components except BPE and the temperature was maintained at 37℃ on a heating stage at atmospheric CO_2. Retinal ganglion cells were easily recognized as cells with long processes. Time-lapse imaging of the FRET indicator was performed on a Leica TSP2 confocal microscope. The indicator was excited at 458nm and dual emission images were collected at 465-490nm (cyan) and 535-560nm (yellow) every 30 seconds. Background was subtracted from both cyan and yellow channel images and the ratios of cyan-to-yellow emissions were then calculated for each time point and normalized to the average emission ratio just before treatment.

Zebrafish maintenance. Wildtype Tü strain zebrafish were raised and maintained according to Mullins et al (Mullins et al., 1994). Zebrafish embryos were staged by time

after fertilization and/or morphology (Kimmel et al., 1995). Heterozygous transgenic Brn3c:GAP43-GFP (Xiao et al., 2005) or Isl2b:GFP fish (Pittman et al., 2008) were used to produce embryos for cell transplantation experiments or in situ hybridization studies. Homozygous zebrafish robo2 *astray*^{te284} or *astray*^{ti272z} mutant fish (Fricke et al., 2001) were mated to produce homozygous mutant embryos for morpholino injections. All fish lines were kindly made available by the Chien laboratory (Utah). The *astray* mutations were identified by PCR as described previously (Chalasani et al., 2007). Fish were raised in 0.006% phenylthiourea to prevent pigmentation in embryonic and larval fish.

Cloning of Zebrafish calmodulin-activated adenylate cyclases. The full-length protein sequences and calmodulin-binding sites of human ADCY1 and ADCY8 were used to BLAST zebrafish genomic and EST databases at the Sanger Centre and NCBI. Two close relatives of ADCY1 and one of ADCY8 were found. Total RNA was extracted from 48hpf zebrafish embryos and reverse-transcribed into cDNA. Primers for RT-PCR were designed according to the zebrafish genomic and EST sequences. The sequences were completed by 5'- and 3'-RACE (Rapid Amplification of cDNA Ends, BD Biosciences, K1802-1). According to the seventh version of the Ensemble zebrafish database (Zv7, release 50; http://www.ensembl.org/Danio_rerio/Info/Index), zADCY1a is located on chromosome 20 while both zADCY1b and zADCY8 are located on chromosome 2.

Constructs. The ICUE3 construct used as a cAMP sensor for FRET imaging was a kind gift from Dr. Zhang (DiPilato and Zhang, 2009). The pHuC:Gal4-VP16 and pUAS:EGFP plasmids were a kind gift from Dr. Jesuthasan (Hendricks and Jesuthasan, 2007). The pUAS:GAP43-mGFP plasmid was made to label axons by replacing the EGFP coding region from pUAS:EGFP with a coding sequence for GAP43-mGFP (Xiao et al., 2005).

The zebrafish ADCY8 expressing plasmid pUAS:ADCY8 was made by replacing the EGFP coding region from pUAS:EGFP with the zADCY8 coding region.

Whole-mount in situ hybridization. In situ hybridization was performed as described (Chalasani et al., 2007) with minor modifications. Briefly, digoxigenin-labeled anti-sense cRNA probes were incubated with embryos to detect the expression pattern of various transcripts. anti-DIG-POD (Roche, Cat#11 207 733 910) was applied and the signal was amplified with a Cyanine 3-coupled tyramide system method (PerkinElmer, NEL 744). Immunostaining was performed simultaneously where needed to detect the co-localization of GFP labeled axons.

Immunostaining. Immunostaining was performed as previously described (Chalasani et al., 2007). Briefly, zebrafish larvae were fixed overnight in 4%PFA and treated with 0.2% collagenase for 2.5 to 3 hours to facilitate antibody access into the tissue. Goat anti-GFP (1:500, Rockland Immunochemicals Inc., Cat# 600-101-215) and anti-goat IgG Alexa Fluor 488 (1:500; Invitrogen) were used to visualize green fluorescent protein (GFP)-positive cells and axons. Immunostained larvae were imaged with a Leica (Wetzlar, Germany) TSP2 confocal microscope and all Z-series stacks are shown as two dimensional projections.

Antisense morpholino design, injection and analysis. Anti-sense morpholinos were synthesized by Gene-Tools, LLC (Philomath, OR). Two were designed to block pre-mRNA splicing and targeted exons within the first guanylate cyclase domain of zADCY8. Both cause shifts in the reading frame and almost immediate truncation of the translated protein. The morpholino sequences were: (E3) 5'-AAG ACA GAA ATT ACC TCA CGT TCT C-3' (underlined nucleotides correspond to exon sequence) and (E4) 5'-AAG TGT

GTT TAC TTA CGT GTG CCA G-3'. The sequence of the standard control morpholino from Gene-Tools is 5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3'. Morpholinos were micro-injected into the yolks of one to two-cell stage embryos immediately after fertilization at the following concentrations: E3: 1.8ng/embryo; E4: 3.2ng/embryo, and E3+E4: 1.0ng E3 + 1.5ng E4/embryo. To characterize the effects of morpholinos on splicing, RT-PCR was performed using cDNA templates prepared from the RNA of 48hpf injected embryos. The PCR products were sequenced to confirm deletion of the expected exons.

Visualizing retinotectal projections. Zebrafish larvae at 5dpf were fixed in 4% paraformaldehyde (PFA) overnight and mounted in 1.3% agarose on glass slides. Retinal axons from the left eye were anterogradely labeled with the lipophilic tracer DiI (D282; Invitrogen) and those from the right eye with DiD (D7757; Invitrogen), both dissolved at 2.5 mg/ml in dimethylformamide. After injection, the dyes were allowed to diffuse overnight at room temperature. The dye-labeled retinotectal axons were scanned on a Leica TSP2 confocal microscope and all images are presented as projections of Z series.

Zebrafish retinal ganglion cell culture. Zebrafish retinal explants were prepared and cultured as previously described (Chalasani et al., 2007). Control or zADCY8 morpholinos were injected into 1-cell stage embryos. At 2dpf, the embryos were anesthetized and the eyes were removed. Retinal explants from zADCY8 and from control morpholino treated fish larvae (approximately 10 retinas per condition) were cultured on opposite halves of poly-lysine (200 mg/ml) and laminin (80 mg/ml) coated glass bottomed MatTek dishes. After 24h in culture, retinal axons were allowed to grow for a 75 minute control period and then for an additional 75 minute treatment period after

the addition of supernatants containing: (1) mock transfected, (2) human slit2-transfected, (3) zebrafish SDF1a-transfected, or (4) human slit2-transfected plus zebrafish SDF1a. Images were taken with a CCD camera (Spot; Phase 3 Imaging, Glen Mills, PA) at the beginning of the control and treatment periods and the end of treatment period. The migration distances of retinal axons during control and experimental periods were analyzed using Image-Pro software.

Cell transplantation. Donor embryos from Brn3c:GAP43-GFP transgenic parents were injected at the 1-cell stage with a mixture of rhodamine-conjugated Dextran (Molecular Probes, D3308) and zADCY8 morpholinos. Host embryos were uninjected wild type and stage-matched with donor embryos. At the sphere stage, embryos were dechorionated and about 20-50 donor embryo blastocysts were transplanted near the animal pole of the host, the region fated to become retina (Kozlowski et al., 1997). The donor embryos were phenotyped at 5dpf and the host embryos were fixed at 4dpf for immunostaining analysis.

Electroporation into the eye. Electroporation of the eye was performed as described with slight modifications (Hendricks and Jesuthasan, 2007). Embryos were injected at the 1-cell stage with zADCY8 morpholinos. At about 24hpf, TE buffer containing the plasmids HuC:Gal4-VP16, UAS:GAP43-GFP, with or without pUAS:zADCY8 were injected into the left eye and subjected to five 13V or 14V pulses 1 millisecond in duration and 500ms apart using an ECM 830 square-wave electroporation system (BTX, Division of Genetronics, Inc., San Diego, CA). The HuC promoter drives gene expression in early differentiated neurons (Park et al., 2000). At 4dpf, the electroporated fish larvae were fixed for immunostaining and analysis.

RESULTS

SDF1 induces a calmodulin dependent increase in cAMP within retinal ganglion cells

Previous studies have defined the outlines of a signaling pathway through which the chemokine SDF1 reduces axonal responses to repellent guidance cues in cultured embryonic primary neurons (Chalasani et al., 2003). This pathway is unusual as it appears to require an increase in cAMP that is induced by a pertussis toxin sensitive $Ga_{i/o}$-protein intermediary. This presumed increase has not been measured directly and contrasts with measured reductions of cAMP levels in SDF1 treated astrocytes and epithelial cells (Dwinell et al., 2004; Warrington et al., 2007). We used the FRET-based cyclic cAMP sensor ICUE3 (Indicator of cAMP using Epac) (DiPilato and Zhang, 2009) to directly detect SDF1 induced changes in cAMP levels in cultured embryonic retinal ganglion cells. An expression vector encoding ICUE3 was transfected into cultured primary chick retinal ganglion cells. The ratio of cyan to yellow fluorescence was monitored to track changes in cAMP levels. This ratio increased in SDF1 treated cells over the course of more than 10 minutes, indicating that cAMP levels rose in response to SDF1 treatment (Figure 1A,B,C). No increase in cAMP was detected without the application of SDF1, and even greater increases were observed in response to application of the adenylate cyclase stimulator forskolin (Figure1D). The SDF1-induced increase in cAMP, but not the forskolin induced increase in cAMP, was abolished by the calmodulin antagonist Calmidazolium (CMZ) (Figure 1E). These findings demonstrate that in primary embryonic retinal ganglion cells, SDF1 can induce an increase in cAMP that is dependent on calmodulin activation. They are consistent with previous findings in embryonic neurons and support the hypothesis that SDF1's effects are mediated through calmodulin activated adenylate cyclases.

The identification of calmodulin activated adenylate cyclases in zebrafish

BLAST searches identified three adenylate cyclases (ADCYs) in the Ensembl zebrafish genome database that contain sequences matching calmodulin binding sites in higher vertebrate ADCY1 and ADCY8 (Vorherr et al., 1993; Gu and Cooper, 1999). The full coding sequences of three zebrafish ADCYs were reconstructed by 5'- and 3'-RACE using 48 hpf zebrafish cDNA as a template. Two of them are most closely related to human ADCY1 and the third to ADCY8, so we have named them zADCY1a, zADCY1b, and zADCY8 respectively (GeneBank accession numbers: ADCY1a, GU169394; ADCY1b, GU169395; ADCY8, FJ472834). The organization of human as compared to zebrafish exons and introns, guanylate cyclase domains, and calmodulin binding sites are all highly conserved (Figure 2A,B and Figure S1A,B,C). The amino acid sequences of the zebrafish guanylate cyclase domains are approximately 90% identical to those in the corresponding human ADCYs.

This study focuses on the functional characterization of zADCY8 during retinal axon outgrowth. The predicted gene structure of zADCY8 is strikingly similar to that of hADCY8. Both have 18 exons. The numbers of nucleotides in each exon are similar between fish and humans except for the first and last exons, which are both shorter in zebrafish. We have tentatively numbered the exons in zADCY8 according to their human counterparts. There are two calmodulin-binding domains, one near the N-terminus and the other near the C-terminus. Both are required for calmodulin regulation of enzymatic activity (Gu and Cooper, 1999; Simpson et al., 2006). The N-terminal most calmodulin-binding site is thought to recruit calmodulin to ADCY8 while the interaction of calmodulin with the C-terminal most site is thought to stimulate ADCY8 cyclase activity (Simpson et al., 2006). Both calmodulin binding sites are highly conserved between zebrafish and

higher vertebrates (Figure 2A). There are only a small number of conservative substitutions which would not be expected to interfere with calmodulin-binding (Rhoads and Friedberg, 1997).

ADCY8 is expressed in retinal ganglion cells within the zebrafish retina

In situs for zADCY8 in 36hpf zebrafish embryos demonstrate expression in the CNS as a whole and in the retinal ganglion cell layer of the eye (Figure 2C,D). To confirm that zADCY8 is expressed in retinal ganglion cells, in situs were performed in Isl2b:GFP transgenic zebrafish in which all differentiated retinal ganglion cells (RGCs) express GFP (Pittman et al., 2008). zADCY8 transcripts were detected within individual RGC cells in 36hpf embryos (Figure 2E.F). This is a relatively early time when the first RGCs have been born and their axons are just beginning to cross the ventral midline (Burrill and Easter, 1995). Two non-overlapping RNA probes detected the same expression pattern for zADCY8. zADCY1b, but not zADCY1a, is also expressed at comparable levels and times in retinal ganglion cells (Figure S1E-F). These results demonstrate that the calmodulin-stimulated adenylate cyclases zADCY8 and ADCY1b are expressed in RGCs at a time consistent with their playing a role in retinal axon guidance.

Morpholino design for knockdown of zADCYs

ADCYs require both guanylate cyclase domains for catalytic activity (Willoughby and Cooper, 2007). We designed morpholinos that were predicted to cause disruption of the first guanylate-cyclase domain of zADCY8 by causing early exons to be skipped during the splicing of pre-mRNAs (Morcos, 2007). Skipping these exons should cause a shift in reading frame and premature termination of translation. The efficacy of each morpholino was tested by performing RT-PCR for targeted sequences using cDNA prepared from morpholino injected as compared to control 48 hpf embryos. Morpholinos targeting either

exon 3 or 4 of zADCY8 were tested singly and in combination (Figure 2B). Agarose gel electrophoresis and sequencing of the RT-PCR products indicated that each mopholino induces truncated PCR products of the expected size (Figure 2B, arrow heads), loss of either exon 3 or exon 4 as expected, and generation of an early termination codon in the truncated sequences. Combining the two morpholinos induces a mixture of PCR products missing exon 3, exon 4, or both exons (Figure 2B, arrow heads). Most subsequent knockdown experiments were performed with the two morpholinos together, but where noted, phenotypes were confirmed with single morpholinos. Comparable splice blocking morpholinos against exons 5 or 7 of ADCY1a (Figure S1B) and exons 4 and 7 of ADCY1b (Figure S1C) were made and tested.

Knockdown of zADCY8 attenuates the anti-repellent effect of SDF1a in vitro

We hypothesized that SDF1 induced calmodulin activation stimulates zADCY8 and that the resulting elevation of cAMP causes retinal axons to be relatively insensitive to repellent signals. We therefore tested whether zADCY8 knockdown makes zebrafish retinal ganglion cell axons less responsive to the anti-repellent effects of SDF1a. Retinae were harvested from zADCY8 morpholino or control morpholino injected larvae at 2dpf, cut into small pieces, and cultured on laminin coated glass. Each coverslip had control explants on one side and zADCY8 morpholino containing explants on the other (see methods). Many individual growth cones were photographed at the beginning and end of a 75 minute non-treatment period, the bathing medium was changed, and the same growth cones were photographed again after a second 75 minute treatment period (Figure 3A1-C3). These photographs were used to measure the forward progress of individual growth cones during the two periods. During the treatment period either the repellent slit2, the chemokine SDF1a, or both were added to the cultures.

Knockdown of zADCY8 does not affect the rate of retinal axon outgrowth (Figure 3D-F, compare the average migration distances between control or zADCY8 morpholino treated axons during the non-treatment periods). Retinal axons containing control or zADCY8 morpholino respond equally well to the repellent slit2 (Figure 3D). Retinal axons containing control or zADCY8 morpholinos advance at the same rate in the presence of zSDF1a (Figure 3E). Consistent with our previous findings[4], retinal axons containing control morpholino are significantly less responsive to slit2 in the presence of zSDF1a as compared to its absence (compare empty columns in Figure 3D and 3F). In contrast, retinal axons containing zADCY8 morpholino are equally sensitive to slit2 in the presence of zSDF1a as compared to its absence (compare filled columns in Figure 3D and 3F). The difference is most apparent in experiments where both slit2 and zSDF1 are presented together during the experimental period (Figure 3F). Control morpholino containing retinal axons continue to advance, while zADCY8 morpholino containing axons retract. These findings demonstrate that zADCY8 knockdown makes zebrafish retinal ganglion cell axons insensitive to SDF1a. They are consistent with zADCY8 serving as an essential step in the SDF1a mediated anti-repellent pathway. Importantly, they demonstrate a cell autonomous change in retinal ganglion cell responsiveness to a repellent guidance cue when zADCY8 is knocked down.

Knockdown of zADCY8 induces abnormal ipsilateral retinal axon projections

We examined the consequences of zADCY8 knockdown on axonal pathfinding in the zebrafish retinal projection. After exiting the eye, retinal axons extend through the ventral diencephalon towards the ventral surface of the brain where they cross the midline at the optic chiasm. As they continue to extend, they grow dorsally and posteriorly to the

tectum. In contrast to binocular animals, all retinal axons cross the midline in the zebrafish. We used anterograde dye tracing in 5 dpf zebrafish larvae to compare retinal axon trajectories in zADCY8 morphants. All retinal axons crossed the midline normally and projected to the contralateral tectum in control morpholino-treated larvae (Figure 4A). However, in a significant number of zADCY8 morphants, some or all retinal axons failed to cross the midline and mis-projected instead to the ipsilateral tectum (Figure 4B-D). Abnormal ipsilateral projections were detected in 62% of E3 morpholino treated eyes and in 12% of E4 morpholino treated eyes (Figure 4E). Combining the E3 and E4 morpholinos, using reduced amounts of each that individually produced no ipsilateral mis-projections, produced robust ipsilateral mis-projections. Synergy between the E3 and E4 morpholinos is consistent with their inducing their effects by acting on a common target. This combination of morpholinos is less likely to induce off-target effects than higher concentrations of single morpholinos and was therefore used in most subsequent experiments. Although mis-projecting retinal axons did not cross the midline, they joined the normal ipsilateral optic tract and projected with grossly normal retinotopy into the ipsilateral tectum (Figure S2).

Knocking down zADCY8 caused a marked reduction in the overall size of the embryo (Figure S4E). This effect could in principle be caused by the knockdown of zADCY8 itself since it is widely expressed, especially in the central nervous system, or by a toxic reaction to morpholinos that induces p53 mediated apoptotic cell death (Robu et al., 2007). We co-injected a p53 morpholino and zADCY8 morpholinos together in order to reduce potential p53 mediated apototic cell death (Robu et al., 2007) and found that the eyes and head were still small in morphant as compared to wildtype embryos. Ipsilateral retinal mis-projections were observed at comparable frequencies in embryos containing

ADCY8 or concurrent ADCY8 and p53 morpholinos (data not shown). Knockdown of zADCY8 also induces a developmental delay in the eye. The initial formation of the optic chiasm, which occurs around 36hpf in normal embryos, did not occur until 18hrs later in zADCY8 morphants. The delay appeared to be greater in the eye as compared to the trunk as judged by the expression pattern of cxcr4b in the lateral line primordium (David et al., 2002) (data not shown). These results suggest that ADCY8 knockdown affects both the size and rate of development of the eye and other central nervous tissues. Subsequent experiments were therefore aimed at determining whether the retinal axon misguidance phenotype is induced directly by the loss of zADCY8 in retinal ganglion cells or as an indirect consequence of developmental perturbations along the retinal pathway.

Potential axon guidance cues and receptors are expressed normally in zADCY8 morphants

Knockdown of zADCY8 might alter retinal responses to midline guidance cues, or alternatively, alter the distributions of those cues at the midline. Since there is a significant developmental delay in zADCY8 morphants, we precisely stage-matched zADCY8 morphants with wildtype embryos according to key morphological markers (Kimmel et al., 1995) and the maturity of retinal axon projections as visualized in Isl2b:GFP transgenics (Pittman et al., 2008). We compared a number of midline markers and/or potential guidance cues in normal and morphant larvae at the time when the first retinal axons cross the midline equivalent to 36 hpf in untreated embryos. These included the midline morphogen and potential guidance cue sonic hedgehog (Trousse et al., 2001), nkx2.2 (Marcus et al., 1999), sema3d (Sakai and Halloran, 2006), and the likely repellent cues slit1a, slit1b, slit2, and slit3 (Hutson and Chien, 2002; Hutson et al., 2003). We found that each of these signaling molecules is expressed at the midline in

similar patterns in both wildtype and zADCY8 morphant embryos (Figure S3, A-C' and G-J'). We also examined the expression patterns of two receptors expressed in retinal ganglion cells, robo2 and cxcr4b, each of which is required for normal retinal axon guidance (Fricke et al., 2001; Li et al., 2005). Both were detected in the RGC layer and their expression patterns are comparable between wildtype and zADCY8 morphants (Figure S3, E-F'). Another potential retinal axon guidance receptor, neuropilin1a (Liu et al., 2004), also maintains a normal expression pattern in zADCY8 morphants (Figure S3, D-D'). All of these key cues and receptors are expressed in their appropriate patterns at the actual time that retinal axons first cross the ventral midline. What is more, their normal expression suggests that midline tissues differentiate normally. The errors we see in retinal pathfinding are therefore difficult to explain by the disruption of midline tissues, or from the loss of known midline guidance cues or their receptors.

Transplantation studies support a cell autonomous requirement for zADCY8 in retinal ganglion cells

We took two independent approaches to determine whether knockdown of zADCY8 alters retinal axon responses to midline guidance cues. In the first, we tested whether retinal ganglion cells harvested from zADCY8 morphant embryos and transplanted into untreated wildtype embryos extend axons normally across the ventral midline or whether they mis-project ipsilaterally. Multipotent progenitors were harvested from Brn3c:GAP43-GFP transgenic fish in which a fluorescent axonal tracer is expressed in approximately 50% of cells that differentiate into retinal ganglion cells (Xiao et al., 2005). These donor embryos were injected at the one cell stage with zADCY8 morpholinos and dextran red. Blastocyst cells were harvested from them at 4 hpf and transplanted into stage-matched wild type host embryos near the animal pole (Figure 5A). Chimeric embryos were allowed to grow to 4 dpf. Embryos with GFP labeled retinal ganglion cells in only one

eye were selected using a dissecting fluorescent microscope for analysis. GFP staining was enhanced by immunofluorescence and examined with a confocal fluorescent microscope. Transplanted zADCY8 morpholino containing retinal ganglion cell axons with ipsilateral mis-projections were detected in 7 of 39 embryos (Figure 5C,D). Since only a very small proportion of retinal neurons derive from the transplant, and since most axons project normally even when all retinal neurons contain zADCY8 morpholino, this approach would be expected to underestimate the rate at which mis-projections occur when morpholino containing retinal axons extend in normal tissues. Transplanted retinal axon trajectories that did not contain zADCY8 morpholino projected normally across the midline in 32 of 32 embryos (Figure 5B). These results are significant to the $p<0.05$ level (Fisher's exact test) and are consistent with a retinal ganglion cell autonomous requirement for zADCY8 for normal midline crossing. Further, they strongly suggest that ipsilateral mis-projections in morpholino treated embryos do not arise from a general delay in embryonic development, since ipsilateral projections occured in chimeric embryos in which the overall rate of development was normal. However, since a small number of scattered dextran-labeled transplanted cells were generally present near the ventral midline in chimeric embryos, these results do not absolutely rule out the possibility that morpholino treated midline cells contribute to the formation of ipsilateral misprojections. We were unsuccessful in attempts to transplant wildtype blastocyst cells into zADCY8 morpholino treated embryos, as the recipient embryos were too fragile to survive the transplantation procedure.

Localized expression of full length zADCY8 within the eye rescues ipsilateral mis-projections of retinal axons in zADCY8 morphants

We used a second approach to confirm that zADCY8 is required within retinal ganglion cells for normal pathfinding. Full length zADCY8 was transfected into retinal neurons of

morpholino treated embryos to determine whether its expression rescues midline guidance errors. zADCY8 morpholino treated 24 hpf embryos were injected in one eye with a combination of expression plasmids containing either HuC:Gal4-VP16 and UAS:GAP43-GFP; or with HuC:Gal4-VP16, UAS:GAP43-GFP, and UAS:zADCY8 plasmids. The HuC promoter drives expression in neurons just before they extend axonal processes (Park et al., 2000). The injected eye was immediately electroporated to transfect the DNA into retinal neurons (Figure 6A). Expression of electropporated constructs was detected in only a small number of retinal ganglion cells. In one experiment in which we quantified expressing cells, only an average of 2.2 ganglion cells expressed the control GFP construct and 2.2 cells expressed ADCY8. Ipsilaterally mis-projecting retinal axons were detected in 14% (8 of 56) of zADCY8 morphant embryos expressing only the control GFP construct (Figure 6B,D). This low frequency of ipsilateral mis-projections can be explained by the low proportion of retinal neurons that were labeled, since most retinal axons project contralaterally even in morphant embryos. Ipsilaterally mis-projecting axons were detected in only 4% (3 of 75) of morphant embryos in which full length zADCY8 was transfected into the retina (Figure 6C,D). Expression of full length zADCY8 in non-morphant embryos did not affect retinal midline crossing. Thus, expression of full length zADCY8 rescued ipsilateral mis-projections in morphant embryos. These results argue strongly that ipsilateral mis-projections are not caused by a general delay of development in morphant embryos since rescue was successfully accomplished without reversing the overall developmental delay. They support the idea that zADCY8 activity within retinal ganglion cells influences the behavior of their axons at the midline.

zADCY8 works together with zADCY1b to facilitate retinal midline crossing

We tested whether the calmodulin activated cyclases zADCY1a or zADCY1b contribute to normal guidance of retinal axons at the midline. Multiple splice-altering morpholinos were confirmed to induce premature stop codons within the first of their two cyclase domains (Figure S1B,C). As expected from the absence of zADCY1a expression in retinal ganglion cells, zADCY1a morpholinos, even at excessive concentrations that caused approximately 50% mortality and induced reduced eye and brain size in the surviving embryos (Figure S4C), produced almost no ipsilateral misprojecting retinal axons (Figure S4H,K). The same was true for a morpholino targeting sema3a1 (Figure S4B,G,K). In contrast, morpholinos directed against ADCY1b did induce ipsilateral retinal misprojections in 22% of morphants (Figure S4I,K). Since zADCY1b and zADCY8 morphants phenocopied one another, we next tested whether these two cyclases act cooperatively to promote midline crossing. Combining morpholinos to each at doses too low to induce ipsilateral misprojections on their own, induced ipsilateral misprojections in 28% of morphants. These findings suggest that the calmodulin activated cyclases zADCY8 and ADCY1b work together to promote retinal axon crossing of the ventral midline.

Knockdown of zADCY8 rescues retinal projection errors in astray mutants with partial loss of robo2 function

Slit is a repellent for retinal ganglion cell axons whose expression near the ventral midline helps determine where the optic chiasm forms (Erskine et al., 2000; Niclou et al., 2000; Hutson and Chien, 2002; Plump et al., 2002; Chalasani et al., 2003; Kreibich et al., 2004; Chalasani et al., 2007). Robo2 is the only slit receptor expressed in retinal ganglion cells as their axons cross the ventral midline in zebrafish (Lee et al., 2001). Zebrafish that have a partial loss of robo2 function display a number of retinal guidance errors, some of which can be rescued by interrupting SDF1a signaling (Chalasani et al.,

2007). If zADCY8 is an essential component within the SDF1 signaling pathway, we predicted that its knockdown should tend to rescue the partial loss of robo2 phenotype. There are several retinal axon pathfinding errors in partial loss of robo2 *astray*^{te284} mutants, including extra midline crossings, anterior and posterior mis-projections, and some ipsilateral mis-projections (Figure 7A) (Fricke et al., 2001). Knocking down SDF1a or its receptor CXCR4b partially rescues the anterior mis-projections in *astray*^{te284} mutants (Chalasani et al., 2007). We focused on this specific class of pathfinding errors when we assessed the effect of zADCY8 knockdown on *astray* fish. We found that the injection of zADCY8 morpholinos into *astray*^{te284} mutant embryos induces significant rescue of anterior mis-projections (Figure 7B and C).

Knockdown of zADCY8 does not rescue retinal projection errors in astray mutants with full loss of robo2 function

Neither SDF1a or CXCR4b knockdown rescues anterior mis-projections of retinal axons in zebrafish that lack functional robo2 altogether, presumably because there is no residual slit/robo signaling that can be strengthened by reducing SDF1 signaling (Chalasani et al., 2007). If zADCY8 is a key step in the SDF1 signaling pathway, then zADCY8 knockdown would not be expected to rescue anterior mis-projections in mutants with a complete loss of robo2. In robo2 null mutants, such as *astray*^{ti272}, retinal axon pathfinding errors are more severe than in *astray*^{te284} mutant embryos (Figure 7D) (Fricke et al., 2001). zADCY8 knockdown has no significant effect on anterior mis-projections of retinal axons in *astray*^{ti272} mutant embryos (Figure 7E and F). RT-PCR was performed to confirm that morpholinos knocked down zADCY8 in both *astray*^{te284} and *astray*^{ti272} mutants (data not shown).

Knockdown of zADCY8 induces fewer ipsilateral retinal mis-projections in mutants with either partial or complete loss of robo2 function

Since ADCY8 is part of a signaling pathway that reduces axonal responses to repellents, its knockdown would be expected to make retinal axons more sensitive to repellent cues. Ipsilateral misprojections in zADCY8 morphant embryos could potentially be explained by retinal axon hypersensitivity to repellents at the midline. Slits are potent repellents which are expressed at the midline and play an important role guiding retinal axons (Erskine et al., 2000; Niclou et al., 2000; Hutson and Chien, 2002; Kreibich et al., 2004; Chalasani et al., 2007). We therefore tested whether reducing or eliminating slit signaling in robo2 mutants reduces ipsilateral misprojections in zADCY8 morphants. We limited our analysis to strong ipsilateral misprojections in morphant embryos, defined as >50% of retinal axons projecting ipsilaterally, since smaller ipsilateral misprojections sometimes occur in robo2 mutants. Morpholino-induced strong ipsilateral mis-projections are less prevalent in either *astray*^{te284} mutants that have a partial loss of robo2 or in *astray*^{ti272} mutants that have a complete loss of robo2 as compared to wildtype embryos (Figure 8D). These observations are consistent with the idea that zADCY8 is part of a signaling pathway that antagonizes slit/robo mediated repellent signaling.

DISCUSSION

Our studies reveal an essential role for calmodulin-activated adenylate cyclases in early retinal pathfinding in the zebrafish embryo. Morpholino knockdown of zADCY8 induces a significant number of retinal ganglion cell axons to be deflected away from the ventral midline instead of following their normal trajectory to the contralateral tectum (Figure 4). Deflected axons generally join the ipsilateral optic tract and travel to the ipsilateral tectum, but in some instances, they travel to entirely inappropriate anterior locations. A second calmodulin activated cyclase, ADCY1b, acts cooperatively with zADCY8 to facilitate midline crossing of retinal axons (Figure S4). The pathfinding errors observed in ADCY8 knockdown embryos arise, at least in part, by a cell autonomous requirement for

ADCY8 within retinal ganglion cells. This was demonstrated in three ways. First, ipsilateral misprojections arise from axons of retinal neurons containing zADCY8 morpholino that are transplanted into untreated wildtype embryos (Figure 5). This indicates that knockdown axons make errors even when interacting with normal host tissues. Second, errors in retinal projections in morpholino treated embryos were rescued by the re-expression of morpholino insensitive zADCY8 in retinal neurons (Figure 6). This demonstrates that zADCY8 expressing axons are more likely to navigate correctly when confronted with knockdown tissues than zADCY8 knockdown axons. Third, experiments *in vitro* demonstrated that knocking down zADCY8 makes retinal ganglion cells relatively insensitive to SDF1a. This shows that zADCY8 is required within retinal ganglion cells for SDF1 signaling. These findings do not preclude a non-autonomous contribution of zADCY8 to retinal axon pathfinding *in vitro*, but they provide decisive support for a retinal ganglion cell autonomous contribution to normal guidance.

Previous studies have suggested that ADCY1 and ADCY3 play important roles in determining where axons terminate within their targets, but have not reported pathfinding errors en route to their targets. Retinal projections have been shown to be perturbed in ADCY1 mutant mice (Welker et al., 1996; Abdel-Majid et al., 1998). Both the orderly retinotopic mapping of retinal projections and the normal separation of ipsi and contralateral axons are significantly disrupted in the lateral geniculate of the thalamus (Ravary et al., 2003; Nicol et al., 2006). These alterations in sensory projections have been interpreted as resulting from ACDY1's presumptive role in activity dependent remodeling of sensory connectivity (Ravary et al., 2003; Nicol et al., 2006). Our results suggest the alternative possibility that guidance errors contribute to some of the mapping errors observed in ADCY1 knockout mice. Abnormal ipsilateral trajectories in the visual

system, like those in zADCY8 morphant fish, could lead to the appearance of abnormal segregation of ipsi and contralateral projections in the geniculate.

An adenylate cyclase that is indirectly regulated by calmodulin (Wei et al., 1996), ADCY3, has recently been shown to be essential for the normal convergence of olfactory sensory cell axons into specific glomeruli in the main olfactory bulb. ADCY3 is expressed in sensory neurons within the olfactory epithelium (Wong et al., 2000). The axons of many olfactory sensory neurons mis-project to multiple abnormal locations in the bulb in ADCY3$^{-/-}$ mice, and single glomeruli form aberrantly from sensory axons expressing dissimilar olfactory receptors (Chesler et al., 2007; Col et al., 2007; Zou et al., 2007). Guidance of olfactory axon projections in ADCY3 mutant mice may be disrupted by perturbations in cAMP levels that affect how axons respond to guidance cues (Song and Poo, 1999; Chalasani et al., 2003). This could occur if cAMP levels control the expression levels of guidance cue receptors on the surfaces of olfactory axons (Imai et al., 2006). These previously reported roles of ADCY1 and ADCY3 demonstrate their role in determining where axons terminate within their targets. Our results are the first to demonstrate that a calmodulin activated ADCY is involved in primary axon guidance as axons extend towards their target.

What accounts for the abnormal deflection of retinal axons away from the ventral midline in zADCY8 knockdown embryos? Our findings argue that ADCY8 is required in retinal ganglion cells, and errors arise from either a diminution of retinal sensitivity to midline attractants, the potentiation of retinal responsiveness to midline repellents, or both. Previous work has shown that the chemokine SDF1 makes chick retinal axons less sensitive to multiple repellents *in vitro*. This effect is mediated by a pertussis toxin and calmodulin-sensitive elevation of cAMP and the activation of PKA (Chalasani et al.,

2003). We hypothesized that calmodulin-activated ADCYs are key components of this anti-repellent signaling pathway. If true, then the knockdown of zADCY8 should make zebrafish retinal axons less responsive to SDF1 and more sensitive to slits. Consistent with this prediction, retinal axons harvested from zADCY8 morphants retract in response to a mixture of SDF1 and slit2 much more strongly than retinal axons injected with a control morpholino (Figure 3). These results show that retinal axons in zADCY8 morphants are insensitive to the anti-repellent effects of SDF1, and that this change is cell autonomous.

zADCY8 knockdown makes retinal axons less responsive to SDF1, and consequently more sensitive to slit, *in vitro*. Can antagonism between SDF1 and slit signaling be observed *in vivo*? In a previous study we showed that retinal axons extend ectopically towards an anterior region of slit expression in mutant fish in which slit/robo signaling is reduced. Further, we found that knocking down SDF1/CXCR4 signaling tended to prevent abnormal anterior extension in mutants with reduced robo2, but not in mutants lacking robo2 altogether (Chalasani et al., 2007). Rescue of the anterior mis-projection therefore requires the presence of some robo2 activity. These results suggest that SDF1/CXCR4 signaling antagonizes slit/robo2 signaling and that residual slit/robo2 signaling is boosted by reducing SDF1/CXCR4 signaling. If zADCY8 is a key signaling step in an SDF1 triggered anti-repellent pathway, then zADCY8 knockdown should also tend to rescue anterior mis-projections in mutants that have a partial, but not a total, loss of robo2. This prediction was confirmed by experiment in this study. zADCY8 knockdown rescues anterior retinal misprojections in partial but not complete loss of robo2 function mutants (Figure 7). Both our *in vitro* and our *in vivo* findings therefore support a model in

which zADCY8 constitutes a key intermediate in an SDF1 activated anti-repellent signaling pathway.

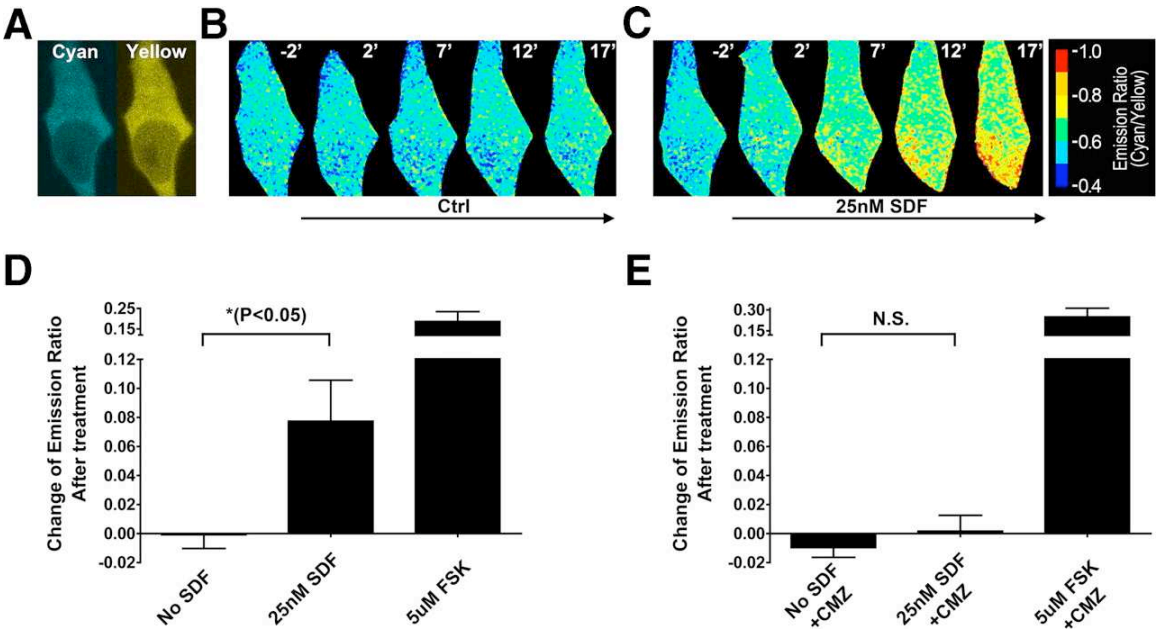
The generalization of this same argument as applied to other potential antagonists of slit signaling at the midline could explain why zADCY8 knockdown induces midline deflection of retinal axons. Slits expressed near the midline play an important role in determining where the optic chiasm forms (Erskine et al., 2000; Niclou et al., 2000; Hutson and Chien, 2002; Plump et al., 2002; Chalasani et al., 2007). Disruptions of slit1 and slit2 in mice induce retinal defasciculation and ectopic crossings at the chiasm (Plump et al., 2002), while loss of the slit receptor robo2 induces widening of the chiasm and ectopic projections of retinal axons to inappropriate targets (Plachez et al., 2008). Retinal axons cross the ventral midline in unusual locations and project ectopically in zebrafish mutants with reduced or missing robo2 (Fricke et al., 2001; Hutson and Chien, 2002). The chiasm normally forms where slit expression is low (Erskine et al., 2000; Niclou et al., 2000; Hutson and Chien, 2002; Plump et al., 2002), but since slits are secreted and are expressed in nearby midline tissues, it is likely that low levels of slit proteins are present even at the chiasm where retinal axons cross. One plausible explanation for ipsilateral misrouting of retinal projections in zADCY8 morphants is that a loss of anti-repellent signaling makes retinal axons hypersensitive to slits in the chiasm.

If zADCY8 knockdown induces abnormal ipsilateral projections by making retinal axons overly sensitive to midline slits, then a reduction in slit/robo signaling would be predicted to rescue these errors. Supporting this idea is the finding that zADCY8 morpholino induced ipsilateral projections are much less frequent in mutant embryos in which robo2 is either reduced or absent. Although our findings are consistent with the idea that ADCY8 knockdown makes retinal axons hypersensitive to midline repellents, we cannot

exclude the alternative possibility that loss of zADCY8 interferes with an attractive mechanism that helps retinal axons cross the midline. Whether zADCY8 is required for an anti-repellent or an attractive response to a midline cue, that cue is unlikely to be SDF1. SDF1 is not expressed at the midline and ipsilateral misprojections are not observed in either SDF 1 or cxcr4b mutants or morphants (Li et al., 2005). Several G-protein coupled signaling pathways have been proposed to affect retinal axon guidance. The adenosine receptor A2b is thought to have a positive influence on cAMP levels in retinal axons that modulates their response to netrin (Shewan et al., 2002), while sonic hedgehog is thought to mediate a repellent response by reducing cAMP levels in retinal growth cones (Trousse et al., 2001). Activation of the metabotropic gluatamate receptor mGluR1 reduces the repellent activity of slit2 on retinal axons in a cAMP-dependent manner (Kreibich et al., 2004). It is not known whether any of these signals influence ADCY1 or ADCY8 activity, nor have any of these GPCRs been reported to affect axonal crossing at the midline.

Studies *in vitro* have demonstrated that modulating cAMP levels within the growth cone can have a profound effect on axon guidance, but the role of cAMP in axonal pathfinding *in vivo* has been less well studied. Our findings demonstrate an essential and previously unappreciated role for calmodulin activated adendylate cyclases ADCY1b and ADCY8 in retinal axon pathfinding *in vivo*. Further, they show that ADCY8 is part of a signaling pathway that antagonizes repellent signaling. They support the idea that retinal axon sensitivity to midline guidance cues is regulated by cAMP levels. Future studies will focus on the identification of additional cues, besides the chemokine SDF1, that influence axonal pathfinding through the activation of ADCY8.

Figure 2.1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Yellow
B
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2'
7'
12'
17'
Ctrl
C
-2'
2'
7'
12'
17'
-1.0
-0.8
-0.6
-0.4
Emission Ratio (Cyan/Yellow)
25nM SDF
D
Change of Emission Ratio After treatment
*(P<0.05)
No SDF
25nM SDF
5uM FSK
E
Change of Emission Ratio After treatment
N.S.
No SDF +CMZ
25nM SDF +CMZ
5uM FSK +CMZ

Figure 2.1. SDF induces a calmodulin-dependent increase in cAMP levels in retinal ganglion cells.

(A) Example images of a chick retinal ganglion cell transfected with ICUE3, a FRET based cAMP biosensor, in the cyan and yellow channels. (B, C) Pseudocolored cyan/yellow ratio images depict FRET responses to (B) vehicle or (C) SDF treatment. Treatment commenced at 0 and images are presented at the indicated time points in minutes. (D) The average changes in the cyan/yellow ratio are indicated for 12 retinal ganglion cells treated first with SDF and then later with forskolin (FSK). The ratios were calculated 15 minutes after the commencement of treatment. The SEMs are indicated. There was a statistically significant difference between the change in FRET signals during the vehicle compared to the SDF1 treatment periods. The significance level was determined by the Mann-Whitney Rank Test. (E) In 12 different retinal neurons, SDF treatment induced no significant change in FRET when 8 μM of the calmodulin antagonist calmidazolium (CMZ) was present.

Figure 2.2

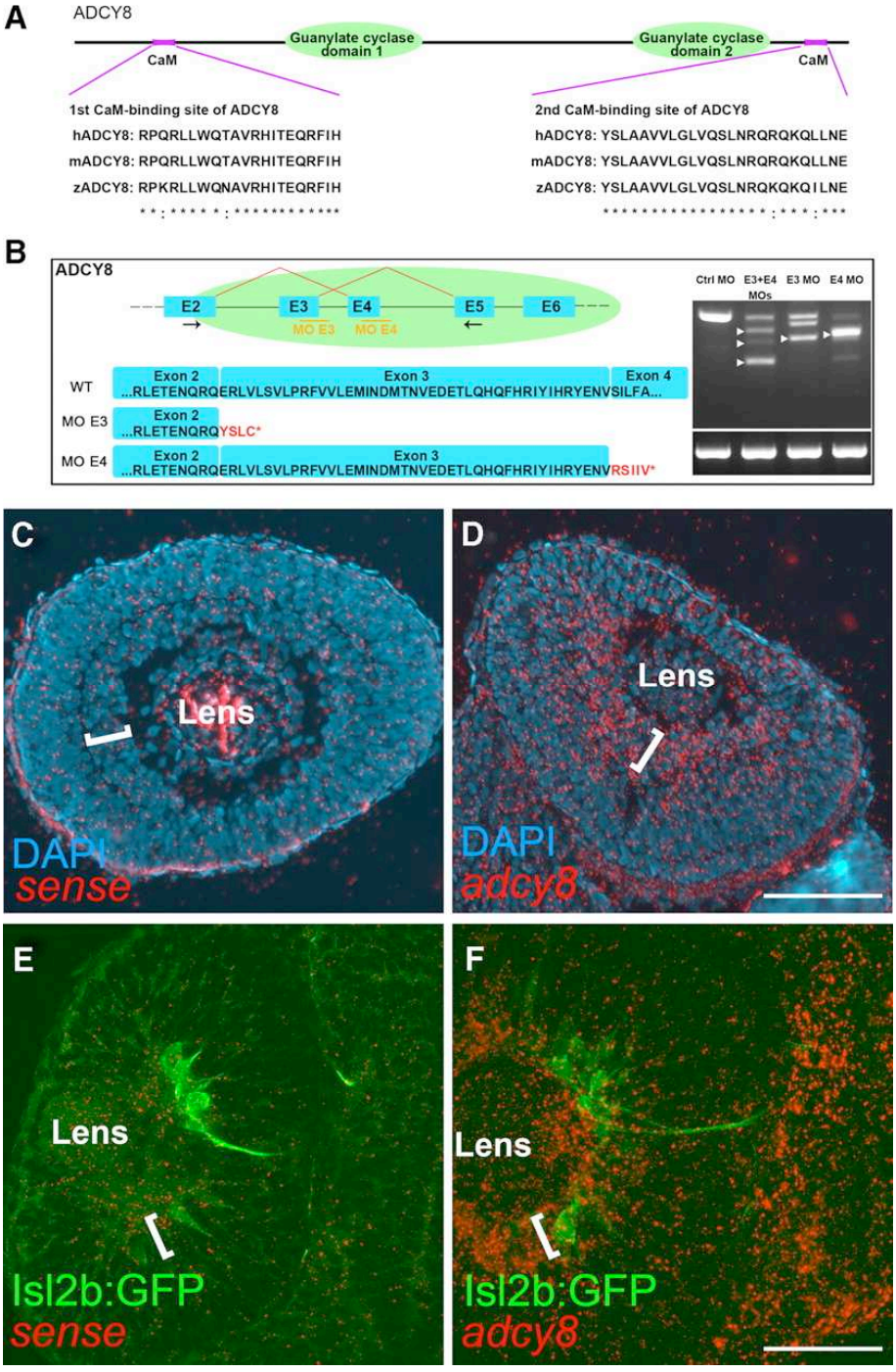
A
ADCY8
Guanylate cyclase domain 1
Guanylate cyclase domain 2
CaM
CaM
1st CaM-binding site of ADCY8
hADCY8: RPQRLLWQTAVRHITEQRFIH
mADCY8: RPQRLLWQTAVRHITEQRFIH
zADCY8: RPKRLLWQNAVRHITEQRFIH
2nd CaM-binding site of ADCY8
hADCY8: YSLAAVVLGLVQSLNRQRQKQLLNE
mADCY8: YSLAAVVLGLVQSLNRQRQKQLLNE
zADCY8: YSLAAVVLGLVQSLNRQKQKQILNE
B
ADCY8
E2
E3
E4
E5
E6
MO E3
MO E4
Ctrl MO
E3+E4 MOs
E3 MO
E4 MO
WT
Exon 2
...RLETENQRQERLVLSVLPRFVVLEMINDMTNVEDETLQHQFHRIYIHRYENVSILFA...
Exon 3
Exon 4
MO E3
Exon 2
...RLETENQRQYSLC*
MO E4
Exon 2
...RLETENQRQERLVLSVLPRFVVLEMINDMTNVEDETLQHQFHRIYIHRYENVRSIIV*
Exon 3
C
Lens
DAPI
sense
D
Lens
DAPI
adcy8
E
Lens
Isl2b:GFP
sense
F
Lens
Isl2b:GFP
adcy8

Figure 2.2 The identification of type 8 adenylate cyclase (ADCY8) in zebrafish and its expression in the retinal ganglion cell layer of the eye.

(A) A close relative of human ADCY8 was identified and cloned in zebrafish. Two sequences in zADCY8 correspond to known calmodulin-binding domains in mammalian cyclases (purple) and are highly conserved between higher vertebrates and zebrafish. (B) Two morpholinos were designed to block the splicing of zADCY8 pre-mRNAs. Morpholino target sequences (orange) correspond to junctions between introns (black lines) and exons (numbered blue boxes). RT-PCR between the primers indicated with black arrows was used to detect morpholino induced mRNA mis-splicing. Normal zADCY8 sequence is represented in black lettering and truncated sequences induced by misplicing in red. Premature stop codons are indicated with asterisks. Green ovals indicate the catalytic guanylate cyclase domains in zADCY8. The highest molecular weight bands generated by RT-PCR in the presence of control morpholinos arise from normally spliced transcripts, while lower molecular weight bands marked with arrowheads arise from morpholino induced mis-splicing. PCR products generated using primers recognizing EF1a in the same samples are shown below. (C-F) zADCY8 is expressed in the retinal ganglion cell layer of zebrafish eyes at 36hpf when the first retinal axons cross the ventral midline. (C, D) Parafin embeded 36hpf embryos were sectioned and stained with DAPI to visualize nuclei. The binding of radio-labeled sense or antisense zADCY8 probes were visualized in a photo-emulsion overlay by dark field microscopy. Specific zADCY8 mRNA expression was detected in the retinal ganglion cell layer (brackets) in the eye. Variable non-specific binding of both sense and anti-sense probes was noted in the lens. (E, F) Wholemount Isl2b:GFP transgenic fish in which retinal ganglion cells are labeled (green) were probed with sense and antisense zADCY8 probes (red). Fluorescent images representing projected Z-stacks 5 μm deep

demonstrate that zADCY8 mRNA is detected within individual retinal ganglion cells (brackets in F) Viewed from the ventral side of the embryo with anterior to the top. Scale bars: 50 mm.

Figure 2.3

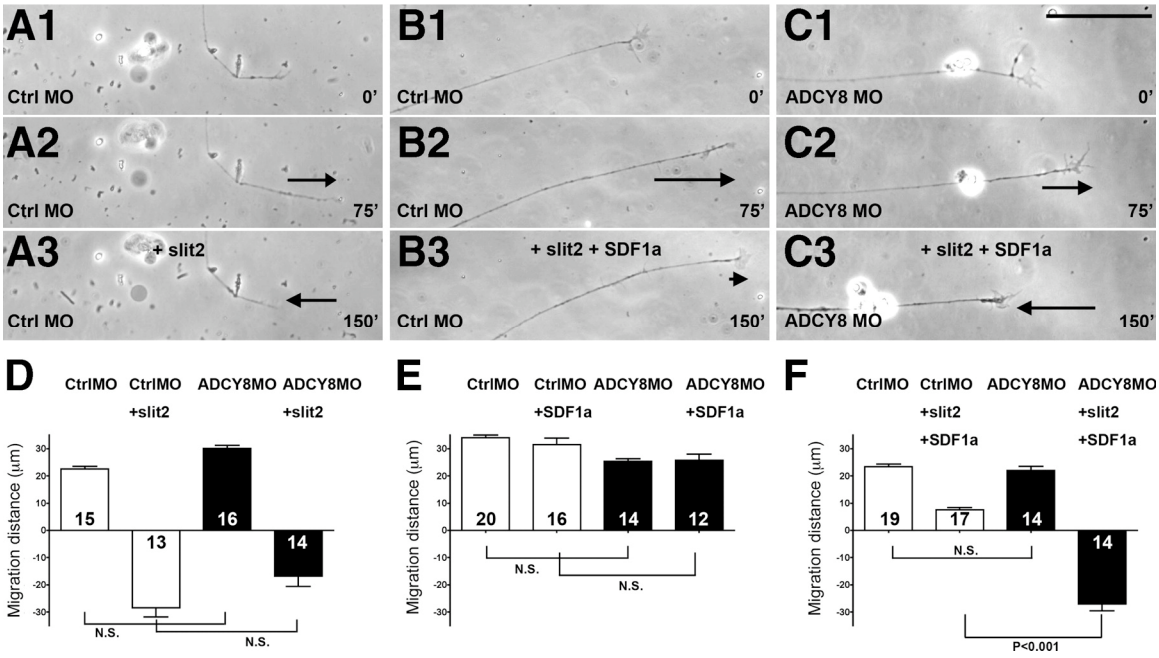
A1
Ctrl MO
0'
B1
Ctrl MO
0'
C1
ADCY8 MO
0'
A2
Ctrl MO
75'
B2
Ctrl MO
75'
C2
ADCY8 MO
75'
A3
+ slit2
Ctrl MO
150'
B3
+ slit2 + SDF1a
Ctrl MO
150'
C3
+ slit2 + SDF1a
ADCY8 MO
150'
D
CtrlMO
CtrlMO +slit2
ADCY8MO
ADCY8MO +slit2
Migration distance (μm)
15
13
16
14
N.S.
N.S.
E
CtrlMO
CtrlMO +SDF1a
ADCY8MO
ADCY8MO +SDF1a
Migration distance (μm)
20
16
14
12
N.S.
N.S.
F
CtrlMO
CtrlMO +slit2 +SDF1a
ADCY8MO
ADCY8MO +slit2 +SDF1a
Migration distance (μm)
19
17
14
14
N.S.
P<0.001

Figure 2.3. Knockdown of zADCY8 blocks the anti-repellent effect of SDF1a *in vitro.*

Retinal explants were prepared from 50hpf control morpholino or zADCY8 morpholino injected embryos and cultured for 24h *in vitro*. Cultures were photographed at the beginning and end of a 75min control period, treated over the course of an additional 75 minute period with either hSlit2, zSDF1a, or both hSlit2 and zSDF1a, and photographed a final time. (A1,2) A control morpholino treated retinal axon advances during the control period and (A2,3) and retracts in response to hSlit2 during the treatment period. (B1-3) A control morpholino treated axon fails to respond to hSlit2 in the presence of zSDF1a. (C1-3) A zADCY8 morpholino treated axon responds to hSlit2 in the presence of zSDF1a. (D-F) Quantitative comparisons of growth cone advance in the presence or absence of hSlit2 and/or zSDF1a. (D) Retinal axons containing the control morpholino (open bars) or the zADCY8 morpholino (filled bars) retract equally well in response to hSlit2. (E) Retinal axons containing the control morpholino or the zADCY8 morpholino do not change their rate of outgrowth in response to zSDF1a. (F) Retinal axons containing the control morpholino do not retract in response to a combination of hSlit2 and zSDF1a, while axons containing the zADCY8 morpholino do retract, showing that retinal axons containing the zADCY8 morpholino are more sensitive to slit2 in the presence of SDF1 than axons containing control morpholino. The number of axons analyzed for each condition is indicated within each bar. The SEM for each condition is indicated by a bar. All experiments were repeated at least three times. Pairwise levels of significsance were estimated using the Mann-Whitney U test. N.S., not significantly different. Scale bar: 50mm.

Figure 2.4

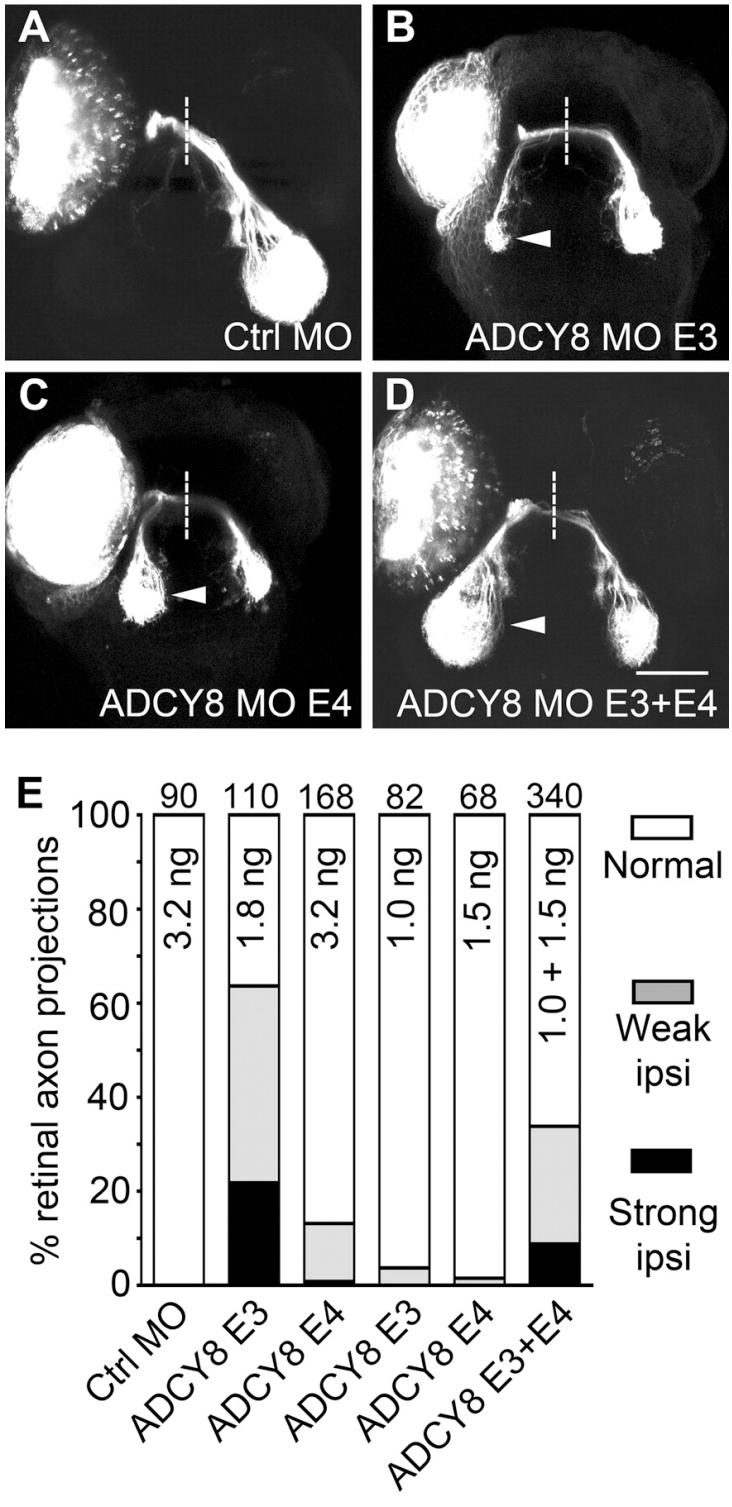
A
Ctrl MO
B
ADCY8 MO E3
C
ADCY8 MO E4
D
ADCY8 MO E3+E4
E
% retinal axon projections
100
80
60
40
20
0
90
110
168
82
68
340
3.2 ng
1.8 ng
3.2 ng
1.0 ng
1.5 ng
1.0 + 1.5 ng
Normal
Weak ipsi
Strong ipsi
Ctrl MO
ADCY8 E3
ADCY8 E4
ADCY8 E3
ADCY8 E4
ADCY8 E3+E4

Figure 2.4. Knockdown of zADCY8 induces ipsilateral mis-projections of retinal axons.

(A-D) Representative examples of retinal axon projections in control and zADCY8 morpholino treated zebrafish larvae. Embryos at the one to two-cell stage were micro-injected with the indicated morpholino, fixed at 5dpf, and retinal axons were labeled with the lipophilic dyes DiI (red) and DiD (blue). All images are dorsal views with anterior to the top. (A) In control morpholino treated larvae, all axons cross the midline and project contralaterally to the opposite tectum. (B-B") In a larvae treated with the E3 zADCY8 morpholino, retinal axons from each eye fail to cross the midline and instead mis-project to ipsilateral tecta. Arrowheads indicate ipsilateral misprojections. In this and subsequent figures a purple hew represents overlapping axons from both contralateral and ipsilateral eyes. (C) Treatment with the E4 zADCY8 morpholino produces similar ipsilateral mis-projections., as does (D) combining low doses of the E3 and E4 zADCY8 morpholinos that by themselves induce few pathfinding errors. (E) The proportions of normal, weak ipsilateral (<50% ispsilateral retinal axons), and strong ipsilateral (>50% ipsilateral axons) projections induced by zADCY8 knockdown are indicated. The amount of each morpholino used per embryo is indicated within each column while the number of eyes examined in each condition is indicated above each column. Scale bar: 100mm.

Figure 2.5

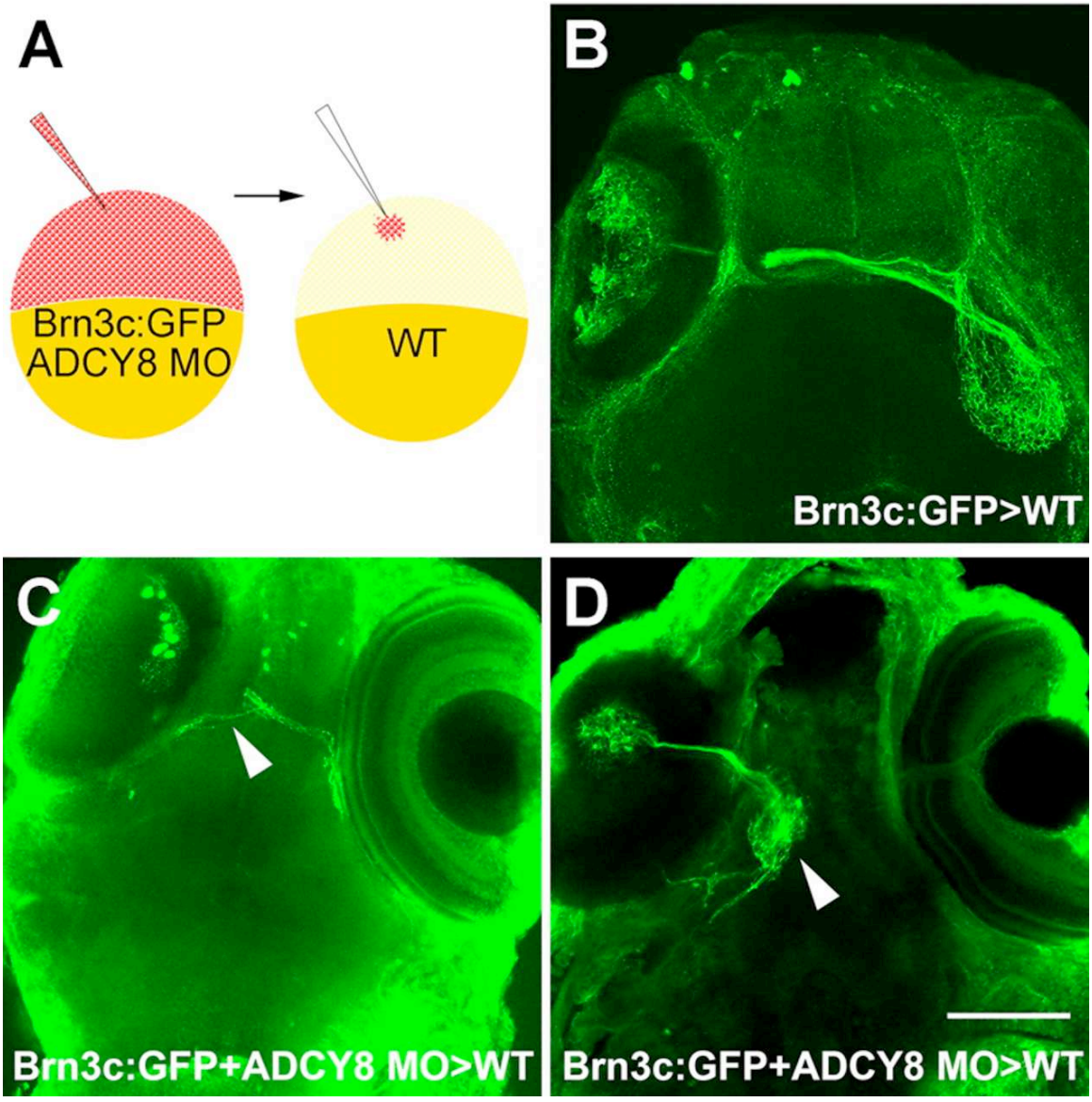
A
Brn3c:GFP
ADCY8 MO
WT
B
Brn3c:GFP>WT
C
Brn3c:GFP+ADCY8 MO>WT
D
Brn3c:GFP+ADCY8 MO>WT

Figure 2.5. Retinal neurons transplanted from morphant embryos into untreated embryos generate ipsilateral mis-projections

(A) Just fertilized transgenic Brn3c:GFP eggs were injected with Dextran Red alone or with Dextran Red and zADCY8 morpholino. Cells were harvested at 4hpf and transplanted into stage matched untreated wildtype host embryos. (B) Transgene labeled transplanted retinal axons containing no morpholino always project contralaterally at 4 dpf. (C,D) Transplanted retinal axons containing zADCY8 morpholino were observed to misproject ipsilaterally in approximately 18% of embryos examined (arrowheads). Scale bar: 100mm.

Figure 2.6

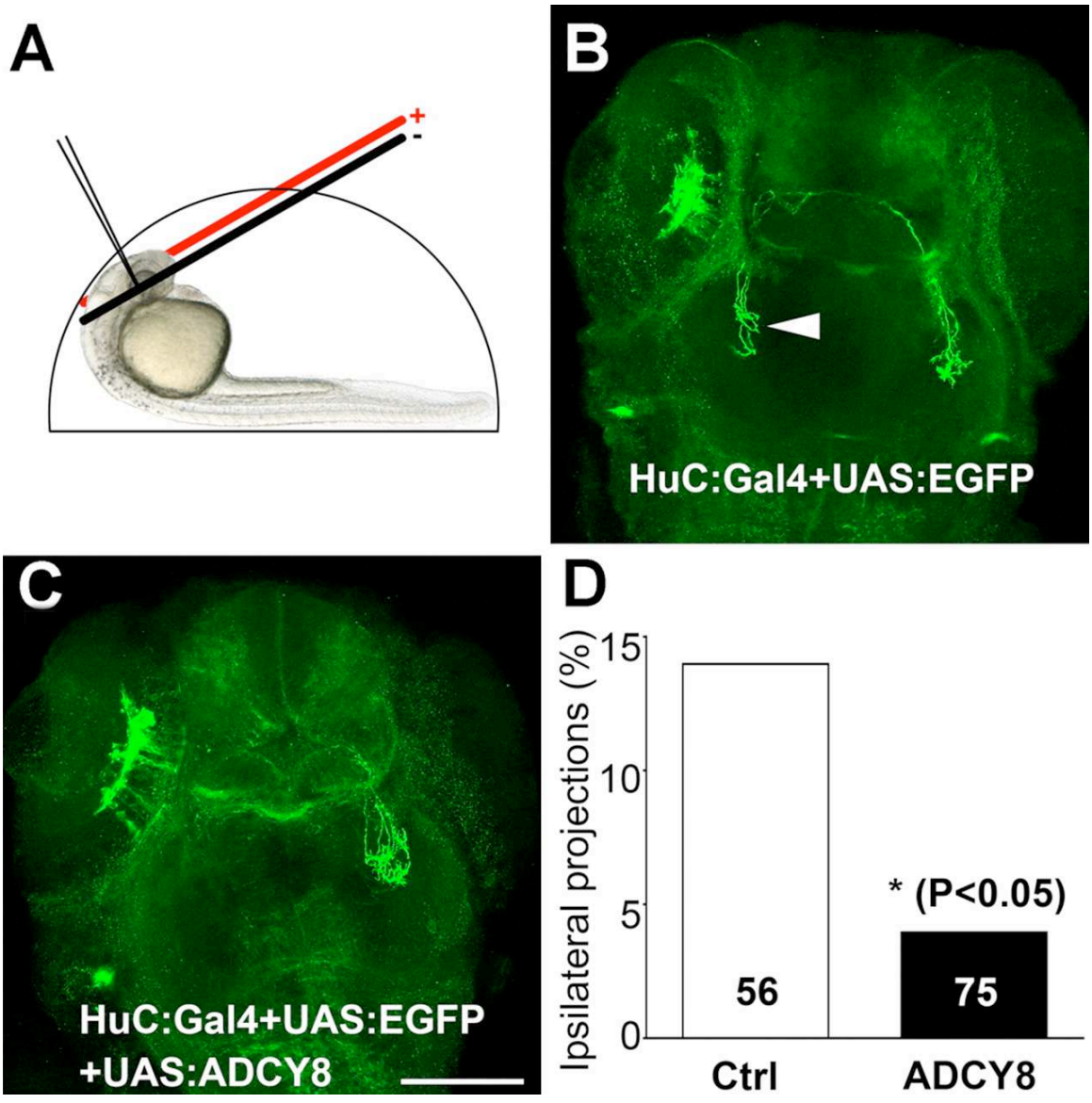
A
+
-
B
HuC:Gal4+UAS:EGFP
C
HuC:Gal4+UAS:EGFP
+UAS:ADCY8
D
Ipsilateral projections (%)
15
10
5
0
56
75
* (P<0.05)
Ctrl
ADCY8

Figure 2.6. Expression of full length zADCY8 in retinal neurons corrects ipsilateral misprojections in zADCY8 morphants.

(A) Either HuC:Gal4 + UAS:GAP43-GFP or HuC:Gal4 + UAS:GAP43-GFP + UAS:ADCY8 were electroporated into one eye of zADCY8 morphant embryos. Embryos were analyzed at 4dpf by immunostaining for GFP. (B) Retinal axons expressing only GFP sometimes mis-project ipsilaterally in ADCY8 morphants. (C) zADCY8 morphants in which retinal axons express GFP and full length zADCY8 rarely mis-project ipsilaterally. (D) zADCY8 expression within the eye significantly reduced the frequency of ipsilateral retinal mis-projections in zADCY8 morphants ($P<0.05$, Fisher's exact test). The numbers in the bars indicate the number of morphant embryos analyzed. The control and ADCY8 plasmids are expressed in similar numbers of retinal ganglion cells in the eye. Scale bar: 100mm.

Figure 2.7

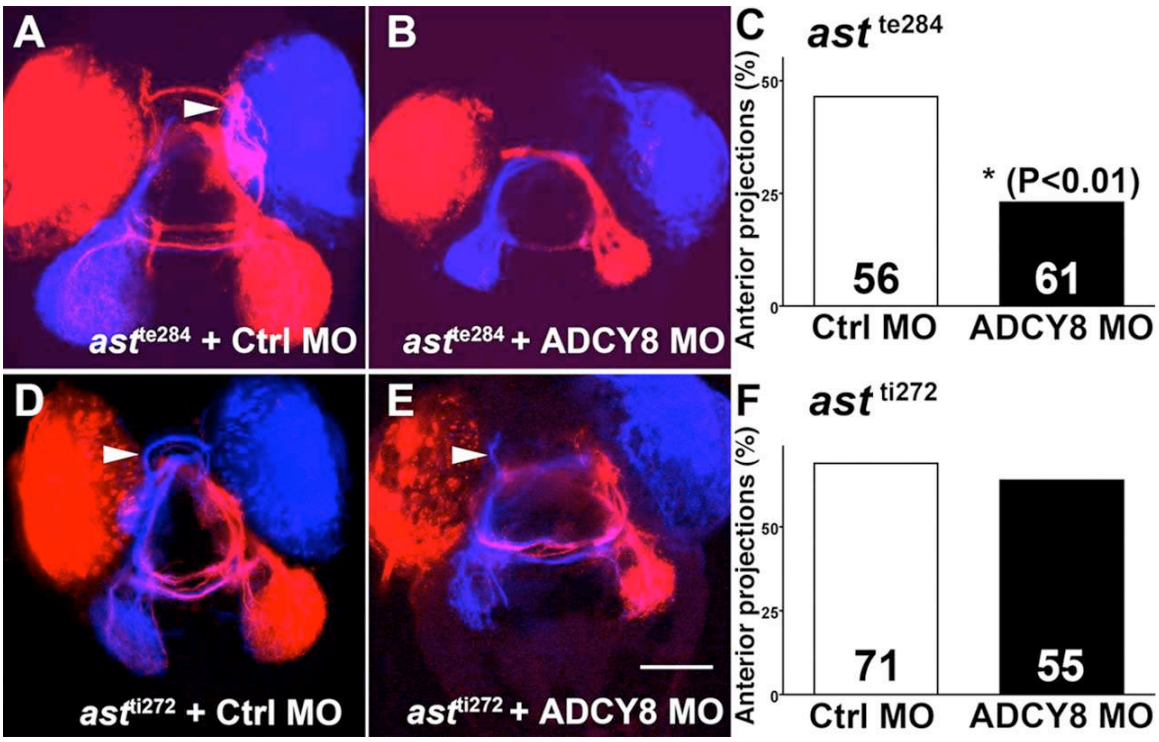
A
B
C
ast te284
Anterior projections (%)
50
25
0
* (P<0.01)
56
61
Ctrl MO
ADCY8 MO
ast te284 + Ctrl MO
ast te284 + ADCY8 MO
D
E
F
ast ti272
Anterior projections (%)
50
25
0
71
55
Ctrl MO
ADCY8 MO
ast ti272 + Ctrl MO
ast ti272 + ADCY8 MO

Figure 2.7. zADCY8 knockdown rescues the anterior mis-projections of retinal axons in mutants with a partial but not a total loss of robo2 function.

Retinal axons project along multiple ectopic pathways in both (A) *astray*^{te284} mutants with a partial loss of robo2 function and (D) *astray*^{ti272} mutants with a total loss of robo2 function. One common error is an ectopic anterior axon projection after retinal axons cross the midline (arrowheads). (B,C) anterior mis-projections are less frequent in zADCY8 morpholino treated 5dpf *astray*^{te284} larvae; but not (E,F) in *astray*^{ti272} larvae. The numbers of eyes analyzed for each condition are indicated within the bars. Fisher's exact test was used to determine levels of significance. Dorsal views with anterior to the top. Scale bar: 100mm.

Figure 2.8

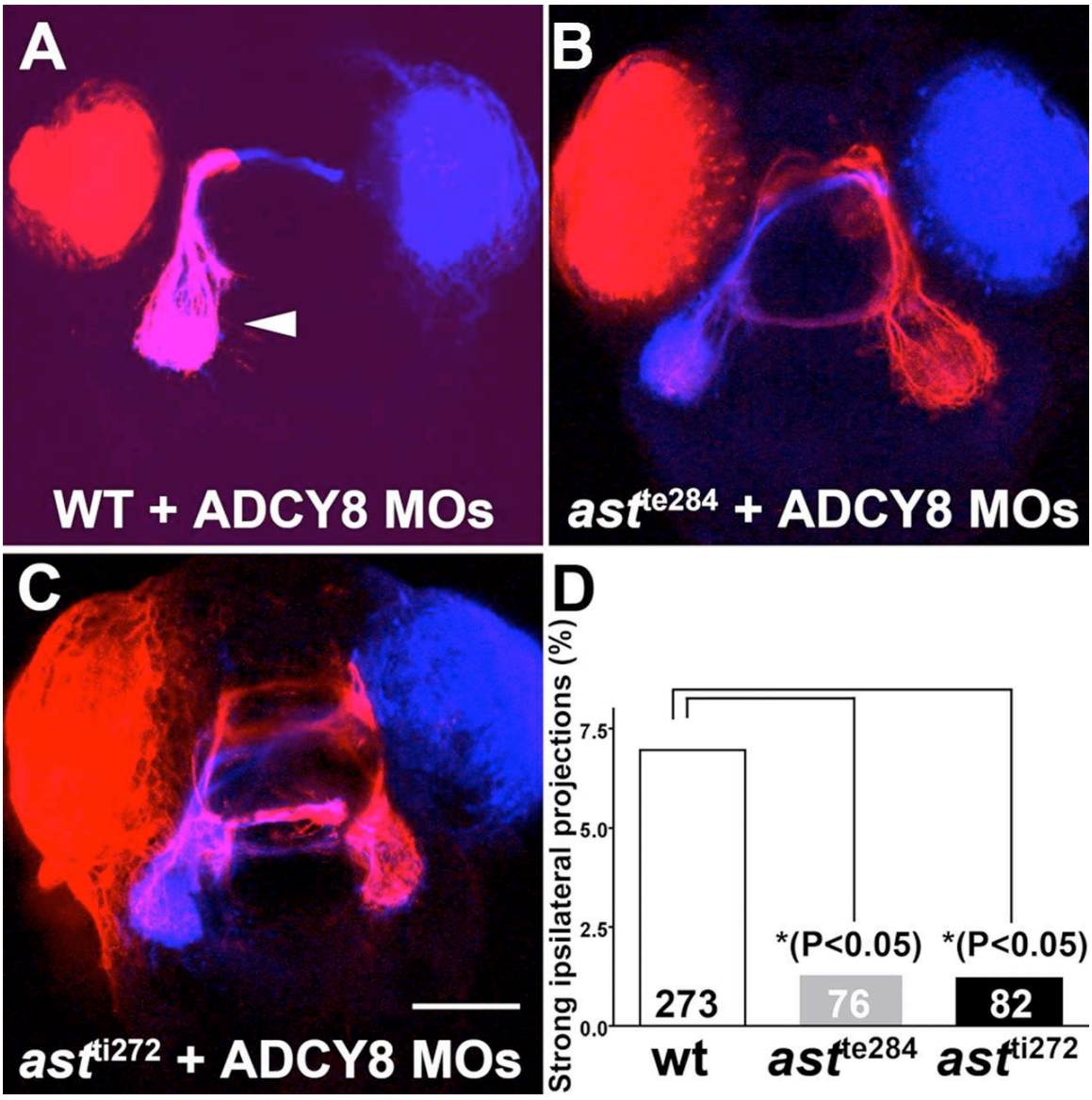
A
WT + ADCY8 MOs
B
ast^te284 + ADCY8 MOs
C
ast^ti272 + ADCY8 MOs
D
Strong ipsilateral projections (%)
7.5
5.0
2.5
0.0
*(P<0.05)
*(P<0.05)
273
76
82
wt
ast^te284
ast^ti272

Figure 2.8. Ipsilateral mis-projections induced by zADCY8 knockdown are rescued in *astray* mutants that have either a partial or total loss of robo2 function.

(A) An example of a strong ipsilateral mis-projection (arrow head) induced by zADCY8 knockdown in a wildtype larvae. Examples of (B) *astray*^{te284} and (C) *astray*^{ti272} larvae treated with zADCY8 morpholino that have weak ipsilateral mis-projections. (D) The percentage of strong ipsilateral mis-projections is significantly reduced in both *astray* mutants as compared to wildtype larvae. The numbers of eyes analyzed for each condition are indicated in the bars. The Fisher's exact test was used to determine levels of significance. Dorsal views with anterior to the top. Scale bar: 100mm.

CHAPTER 3: RETINAL AXON CROSSING AT THE CHIASM REQUIRES CAMP REGULATED SEMAPHORIN SIGNALING

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ABSTRACT

Growing axons navigate through a complex environment as they respond to attractive and repellent guidance cues. Axons can modulate their responses to guidance cues through a G protein-coupled, cAMP-dependent signaling pathway. To examine the role of G protein signaling in axon guidance *in vivo*, we used the GAL4/UAS system to drive expression of dominant negative (DN) heterotrimeric G proteins in retinal ganglion cells (RGCs) of embryonic zebrafish. Retinal axons normally cross at the ventral midline and project to the contralateral tectum. Expression of DNGα_S in RGCs causes retinal axons to misproject to the ipsilateral tectum. These errors resemble ipsilateral misprojections in *adcy1*, *adcy8*, *nrp1a*, *sema3D*, or *sema3E* morphant embryos, and in *sema3D* mutant embryos. *Nrp1a* is expressed in RGCs as their axons extend towards and across the midline. *Sema3D* and *sema3E* are expressed adjacent to the chiasm, suggesting that they facilitate retinal midline crossing. We demonstrate synergistic induction of ipsilateral misprojections between *adcy8* knockdown and transgenic DNGα_S expression, *adcy8* and *nrp1a* morphants, and *nrp1a* morphants and transgenic DNGα_S expression. Using QPCR analysis we show that either transgenic DNGα_S expressing embryos or *adcy8* morphant embryos have decreased levels of *nrp1a* mRNA. Ipsilateral misprojections in either *adcy8* morphants or DNGα_S expressing transgenic embryos are corrected by the expression of a *nrp1a* rescue construct expressed in RGCs. These findings suggest that elevated cAMP levels promote Neuropilin1a expression in RGCs, increasing the sensitivity of their axons to Sema3D and Sema3E at the midline, and consequently facilitating their crossing at the chiasm.

INTRODUCTION

The assembly of functional neural circuits during development requires that neurons interconnect with great specificity. The motile structure at the tip of the growing axon, the growth cone, interprets a constellation of permissive, attractive, and repellent cues that guide it to its target (Tessier-Lavigne and Goodman, 1996). Specific receptors that decorate its surface determine how a growth cone responds to the guidance cues it encounters. G Protein-Coupled Receptors (GPCRs) are specialized to detect signals in the extracellular environment including photons, peptides, and small organic molecules. They are therefore appealing candidates to direct axonal pathfinding. GPCRs are key mediators of chemotactic behavior. Examples include the cAMP sensor in the amoeboid slime mold *dictostylium* and chemokine receptors on leukocytes and migrating germ cells (Klein et al., 1988; Bleul et al., 1996; Knaut et al., 2003). GPCRs are prevalent in the nervous system, including metabotropic neurotransmitter receptors expressed by neurons and odorant receptors in the olfactory epithelium. Odorant receptor dependent cAMP signaling is critical for olfactory sensory neurons to reach their appropriate glomerular targets (Imai et al., 2006; Chesler et al., 2007). The metabotropic $GABA_b$ receptor mediates axonal repulsion *in vitro* (Xiang et al., 2002), and the metabotropic serotonin receptors 5HT1B and 5H51D modulate thalamic axon responses to netrin 1 by inhibiting cAMP production (Bonnin et al., 2007). Sdf1 signaling through the GPCR Cxcr4, or glutamate signaling through the metabotropic mGlur1 receptor, antagonize axonal repellents *in vitro* (Chalasani et al., 2003; Kreibich et al., 2004). While GPCRs are appealing candidate axon guidance receptors, little is known about their role in axonal pathfinding in the intact developing nervous system.

Our objective was to test the contributions of GPCR and G protein mediated signaling to axon pathfinding *in vivo*. To this end, we generated transgenic zebrafish lines expressing dominant negative constructs targeting GPCR effectors, the heterotrimeric G proteins $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{S/olf}$, and $G\beta\gamma$. These dominant negative reagents were specifically expressed in retinal ganglion cells (RGCs) to examine their effect on retinal pathfinding. In zebrafish, RGC axons exit the eye, cross at the ventral midline at the optic chiasm, and then project dorsally and posteriorly to the contralateral tectum and other target locations.

We find that the expression of dominant negative constructs targeting $G\alpha_i$, $G\alpha_q$, or $G\beta\gamma$ induce infrequent and minor errors in retinal projections. In contrast, expression of a dominant negative construct targeting $G\alpha_S$ induces significant ipsilateral tectal misprojections. These errors phenocopy knockdown of the calcium/calmodulin-activated adenylate cyclases *adcy1* or *adcy8* (Xu et al., 2010). Our findings suggest that cAMP levels can regulate midline crossing of RGC axons. Similar ipsilateral misprojections are observed after knocking down semaphorin3D (*sema3D*) or semaphorin3E (*sema3E*), axonal guidance cues which are expressed at the ventral midline, or the semaphorin receptor component neuropilin1a (*nrp1a*) which is expressed in RGCs. Here we show that ipsilateral misprojections induced in retinal axons with impaired cAMP signaling can be ascribed to a concomitant reduction of *nrp1a* expression. We propose that GPCR and Ca^{2+}/calmodulin mediated elevation of cAMP levels promotes the expression of *nrp1a* in RGCs, sensitizes retinal axons to *sema3D* and *sema3E* expressed at the midline, and thereby promotes retinal axon crossing.

METHODS

Generation of dominant negative constructs. DNGα subunit constructs were generated by back translation of the c-terminal 11 amino acid sequences of individual alpha subunits after Gilchrist et al (1999). The 5' primer included Pst1 and a Kozak, sequence, while the 3' primer included a stop and a NotI site. Primers encoding the DN peptides were annealed and cloned into the Pst1/Not1 sites in Tol2 dual UAS vector: UAS:MCS;UAS:GAP43-Citrine (Balciunas et al., 2006; Lakhina et al., 2012). The DNβγ, GRKct in PCDNA3, was a gift from the Albert lab (Ghahremani et al., 1999). The plasmid was digested with Not1/HindIII and the resulting 1.5kb fragment was ligated into the Tol2 dual UAS vector (Lakhina et al., 2012).

Generation of Tol2;Atonal7:Gal4-VP16pA (Ath7:Gal4). 7 kb 5' to Ath7 (previously Ath5) promoter was cut from pAth5:eGFP (a gift from Chi-Bin Chien) with BamH1. This fragment was cloned into the BglII site of the Tol2 plasmid (Balciunas et al., 2006). The Gal4-VP16-pA cassette with Not1 sites flanking the target sequence was generated by PCR. The product was cloned into TOPOII and digested with NotI to generate a 935bp fragment. The fragment was cloned into the NotI site of the Tol2 Ath7 promoter-containing plasmid to generate Ath7:Gal4.

Establishment of zebrafish transgenic lines. One-cell stage embryos were injected with Gal4 or UAS Tol2 constructs and raised to adulthood. Founders were identified by crossing to appropriate reporter lines. Two or more independent lines were generated for each UAS or Gal4 construct (Lakhina et al., 2012). Fluorescent F1 progeny were raised to adulthood and crossed to WT. Subsequent generations of UAS or Gal4 embryos were raised and genotyped by PCR for citrine (UAS lines), Gal4, or by screening for fluorescent fish after crossing to reporter lines. Lines have been assigned in the

zebrafish database as follows: Ath7:Gal4:VP16: Tg(Tol2;Atonal7:Gal4-VP16pA)p203a,b; UAS:dnG$\alpha_{i1/2}$:UAScitrine: Tg(14xUAS:dnGNAI;14xUAS:GAP-CITRINE)p204a,b; UASdnG$\alpha_{q/11}$:UAScitrine, Tg(14xUAS:dnGNAQ;14xUAS:GAP-CITRINE)p205a,b; UAS:dnG$_{\beta\gamma}$;UAScitrine, Tg(14xUAS:GRKCT;14xUAS:GAP-CITRINE)p206a,b; UASdnG$\alpha_{S/olf}$:UAS:citrine, (14xUASdnGNAS;14xUAS:GAP-CITRINE)p208a,b; UAS:citrine Tg(14xUAS:GAP-CITRINE)zf360, UAS:Nrp1a Tg(Tol2:14xUAS:Nrp1a;14xUAS:GAP-CITRINE)p208a,b.

Zebrafish maintenance. Zebrafish (*Danio rerio*) were raised and maintained under standard conditions according to Mullins et al. (1994). Zebrafish embryos were staged by time after fertilization and/or morphology (Kimmel et al., 1995). Transgenic lines and mutant used in this study include: Isl2b:GFP Tg(-17.6isl2b:GAP-GFP)zc20 (Pittman et al., 2008), Isl2b:mCherry (Tg(-17.6isl2b:mCherry-HsHRAS)zc25 (Pittman et al 2008), s11o1t:Gal4 Et(e1b:GAL4-VP16)s11o1t (Scott and Baier 2009) UAS:Citrine Tg(14xUAS:GAP-CITRINE)zf360 (Lakhina et al, 2012). *Sema3D*$^{sa1661/sa1661}$ mutants were obtained through the Zebrafish Mutation Project (Sanger Center). Otherwise experiments were carried out in Tu or TLF WT fish maintained in the fish facility at the University of Pennsylvania. Fish were mated to produce embryos for *in situ* hybridization, morpholino injections, and *in vitro* cell culture. Embryos were raised in 1XE3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4 in deionized water), with 0.006% phenylthiourea added at 24h to suppress pigmentation.

Wholemount Immunostaining. Immunostaining was performed as previously described (Chalasani et al., 2007). Larval zebrafish were fixed overnight with 4% paraformaldehyde in PBS followed by methanol dehydration and permeabilization in ice-

cold acetone. Primary and secondary antibodies were used at the following concentrations to visualize GFP-positive cells and axons: 1:500 goat anti-GFP (Rockland Immunochemicals), 1:500 anti-goat IgG Alexa Fluor 488 (Invitrogen), 1:500 anti-rabbit IgG Alexa Fluor 546 (Invitrogen), and 1:500 anti-mouse IgG Alexa Fluor 546 (Invitrogen). Immunostained larvae were imaged using Leica TSP2 or TSP5 confocal microscopes.

Eye fills. Lipophilic dyes were used to orthogradely label retinal ganglion cell axons as described in Xu, et al. (2010). Briefly, fixed five-day post-fertilization larvae were mounted in 1.2% agarose on glass slides. Retinae were pressure-injected with either DiI or DiD (both from Invitrogen) dissolved in dimethylformamide. Dyes were allowed to diffuse overnight at room temperature or for three hours at 28.5^{0}C. Dye-labeled retinotectal axons were imaged using Leica TSP2 or TSP5 confocal microscopes and are displayed as maximum projected z-stacks.

In Situ Hybridization Single label *in situ* hybridization was performed as described in Chalasani et al. (2007), and double label *in situ* hybridization was performed as described by Brend and Holley (2009). DIG-labeled antisense mRNA riboprobes (1:60) were used to visualize RNA location. After probe application and incubation, anti-DIG-POD or anti-Fluorescien-POD (Roche) was applied, followed sequentially by Fluorescein-coupled tyramide and cyanine 3-coupled tyramide to amplify the fluorescent signals (PerkinElmer). Fish were then immunostained to visualize mCherry- or GFP-positive cells. pBSSKII+ Nrp1a (NM_001040326) probe construct targeting nt 1761-2760 was a kind gift from Wattaru Shoji. Semaphorin probes were as follows: pCRII Sema3D (NM_131048) nt. 1810-2566, pCRII Sema3E (NM_001045084), nt.2200-3020

mRNA injections: A Glu-Glu tagged constitutively active $G\alpha_S$ (Q227L) construct was obtained from the Missouri S&T cDNA resource center. mRNAs were generated using the mMessage mMachine kit and poly(A) tailing kit (Ambion). Protein production from injected mRNA was confirmed by immunohistochemistry using mouse anti-Glu-Glu antibody (Abcam Ab24627).

Morpholino Injections: Morpholinos targeting exons 3 and 4 of *adcy8* were injected as described in Xu and Raper (2010). Sema3D translation blocking *sema3D* MO1 (CATGATGGACGAGGAGATTTCTGCA) and splice blocking *sema3D* MO2 (CACATTCAGTCTGCAGCAAGAGAAA) morpholinos were previously described (Brendt and Halloran, 2006). We designed two *sema3E* splice blocking morpholinos, one targeting exon 5, *sema3E* MO1 (TTGTAGAGATGAACACTTACGGTAG) and one targeting exon 8, *sema3E* MO2 (AAAGAGTGAAGCTCCCCTACCGAGC), both of which are predicted to alter the reading frame and induce an early termination of the encoded peptide. *Nrp1a* MO1 (GAATCCTGGAGTTCGGAGTGCGGAA) blocks translation as previously described by Lee (2002). *Nrp1a* MO2 (AATGTTTTTTCCTTACCCGTTTTGA) targets the splice junction between exons 2 and 3. *Sema3A1* (CTTGTAGCCCACAGTGCCCAGAGCA) and *P53* (GCGCCATTGCTTTG-CAAGAATTG) morpholinos were described previously (Shoji et al., 2003; Robu et al., 2007). Synergistic interactions between morpholinos were assessed using a custom Monte Carlo-based simulation that estimates the probability that two different manipulations are independent and additive when combined.

Electroporations were performed according to Henricks and Jesuthasan (2007). Anesthetized 24hpf embryos were singly embedded in drops of 1.2% low melt agarose (#50302 SeaPrep) containing approximately 0.016% tricaine in electroporation ringers

(180mM NaCl, 5mM KCl 1.8mM $CaCl_2$). DNA solutions containing *HuC*:Gal4-VP16 and either control or UAS:*nrp1a* rescue constructs were co-injected into one eye and electroporated using a BTX ECM830 square wave electoporator set to deliver 5 x 1ms pulses at 30V. The *HuC*:Gal4-VP16 plasmid was previously described by Hendricks and Jesuthasan (2007). *Nrp1a* was first cloned into PCR2.1 and subsequently cloned into the dual UAS vector using EcoRV and NheI sites. After electroporation, embryos recovered in E3 medium and were fixed at 3dpf or 4dpf for analysis. Embryos were immunostained for GFP and axonal projections were evaluated for ipsilateral or contralateral projections by confocal microscopy. To better visualize labeled cell bodies and axonal projections in the electroporated embryos displayed in Figure 4, non-specific background staining was selectively removed from individual optical sections that were out of the plane of focus for retinal processes using imageJ. Maximum projections were then produced from stacks of all the sections together.

qPCR: UAS:DNGα_S transgenics were crossed to s1101t:Gal4 fish (Scott and Baier 2009) to drive pan-neuronal expression of the transgene. Embryos were dissected into heads and tails, heads were stored in RNAlater (Ambion), tails were processed using HotShot, and UAS copy number was assessed in the tail material by SYBR Green (Sigma) QPCR for genomic citrine (5'-GCCCCGTGCTGCTGCCCGAC-3'; 5'-CCCGGCGGCGGTCACGAACT-3'), using a $\Delta\Delta$ct comparison with EF1A (5'GGCAGACCGTTGCTGTCGGCG-3'; 5'-GGTTGGGAAGAACACGCCGCAACCT-3') as an endogenous internal control (Bae et al., 2011). Embryo heads were then pooled by estimated copy number. RNA was extracted with Trizol (Sigma) and used to make cDNA using the SuperScript kit (Invitrogen). qPCR was performed on cDNA from pooled embryos using SYBR Green for *nrp1A* (5'-GCCCAAACATGGACCACACAGGCGG-3';

5'-CCTGGCCCGACACCGACGGAC-3'), *cxcr4* (5'-GGCGGTCGATGCAGTCAGCGGG-3'; 5'-GCGGTCCAGGCTGATGAATCGCAGG-3'), and EF1A (5'-GGGGAGCAGCAGCTGAGGAGTGA-3'; 5'-AGGAGCCCTTGCCCATCTCAGCG-3'). Expression levels of pooled fluorescent and non-fluorescent sibling controls with and without morpholino treatment were compared using a two-tailed Student's t-test.

Eye Removal for optic tract degeneration: *Ath7*:Gal4 fish were crossed to either control UAS:Citrine (Lakhina et al, 2012) or UAS:*nrp1A* (rescue) transgenics injected with *adcy8* morpholino and allowed to develop to 3dpf. Anesthetized Citrine-positive fish were mounted in 1% low melt agarose prepared in E3 medium and one eye was removed using sharpened Tungsten needles. Fish were allowed to recover in E3 medium, and returned to the incubator for 48h to allow optic tract degeneration. Fluorescent axons from the intact eye were then imaged and assessed for ipsilateral or contralateral projections. Statistical comparisons were made using Fisher's Exact test.

RESULTS

RGCs expressing dominant negative $G\alpha_S$ misproject to the ipsilateral tectum.

Activated GPCRs signal through heterotrimeric G proteins composed of $\alpha\beta\gamma$ subunits. Upon activation, GPCRs act as GEFs, changing confirmation to promote the exchange of GDP to GTP on the α subunit, and prompting dissociation of the heterotrimer into an active GTP bound α and the $\beta\gamma$ complex. There are four families of $G\alpha$ subunits: $G\alpha_i$, $G\alpha_q$, $G\alpha_S$ and $G\alpha_{12/13}$. Each $G\alpha$ triggers specific downstream signaling events (Oldham and Hamm, 2008). $G\alpha_S$ is named for its ability to **S**timulate adenylate cyclases (Adcy) to produce cAMP, $G\alpha_i$ canonically **I**nhibits adenylate cyclase production of cAMP, while $G\alpha_q$ and the $G\beta\gamma$ complex can promote calcium signaling through PLC

(Downes and Gautam, 1999; Oldham and Hamm, 2006). We previously identified a G protein-coupled calmodulin, cAMP, and PKA-dependent pathway that reduces axonal response to repellents (Chalasani et al., 2003; Kreibich et al., 2004). Sdf-1 signaling through the GPCR Cxcr4 abrogates Slit-2's repellent effect on cultured retinal axons. This pathway signals through $G\alpha_i$, $G\alpha_q$, and βγ subunits (Twery and Raper, 2011). These findings led us to consider whether GPCRs might act more generally as axon guidance receptors.

To test the contribution of G protein-coupled signaling to axon guidance *in vivo,* we generated and expressed Dominant Negative (DN) constructs targeting Gα subunits $_{i/o}$, $_{q/11}$, and $_{s/olf}$ (Gilchrist et al., 1999, 2002). These constructs encode the last 11 amino acids of each Gα subunit. This C-terminal sequence is divergent among the different Gα subunits and is essential for Gα-specific heterotrimer binding to GPCRs (Gilchrist et al., 2002). The peptides act as competitive inhibitors of endogenous heterotrimers for binding to their cognate GPCRs (Figure 1A; Gilchrist et al., 1999). We also generated a construct targeting the βγ complex (Ghahremani et al., 1999). The DNGβγ we used encodes the C-terminal fragment of an endogenous regulator of G protein signaling, GRK-2 (Koch et al., 1994). GRK-2's pleckstrin homology domain binds and inactivates free Gβγ subunits and is sufficient to block dopamine induced Ca^{2+} release in an *in vitro* assay (Ghahremani et al., 1999). We previously used the same $DNG\alpha_i$, $DNG\alpha_q$, and DNGβγ constructs in primary neuronal culture to block the Sdf1/Cxcr4 signaling pathway (Twery and Raper, 2011).

We adapted this approach to examine the contribution of GPCR signaling to axonal pathfinding during normal development in the zebrafish embryo. Gα terminal sequences are identical in the zebrafish and in higher vertebrates. Dominant negative constructs

were cloned into a Tol2:UAS expression vector in which each construct is driven in tandem with GAP43-Citrine, a membrane-targeted fluorescent label suitable for the visualization of long axonal projections (Figure 1B: Lakhina et al., 2012). Tol2 plasmids containing DNGα_i, DNGα_q, or DNG$\beta\gamma$ constructs were tested and found to block Sdf1 mediated anti-repellent signaling in chick DRG growth cones (data not shown), consistent with our previous findings (Twery and Raper, 2011). We generated an *atonal 7* (*atoh7*) promoter driven GAL4 line that we used to drive UAS constructs in RGCs. *Atonal 7* (previously *atonal 5*) is a BHLH transcription factor required for RGC neurogenesis and is expressed in retinal progenitors and RGCs (Kay et al., 2001; Vetter and Brown, 2001). We focused our analysis on the retino-tectal projection because it is a well-established model of midline axon guidance and a system in which we previously showed that Sdf1 mediates anti-repellent activity (Chalasani et al., 2007; Xu et al., 2010).

RGCs are first born in the ventronasal retina at 30 hours post fertilization (hpf) and extend axons out of the eye and through the optic stalk. They project ventrally, crossing the ventral midline to form the optic chiasm, before projecting dorsally and posteriorly to their synaptic targets in the tectum and arborization fields 1-9 (Burrill and Easter, 1994). Retinal axons first cross the midline around 36hpf, sometimes making minor errors that are largely corrected by 3dpf (Hutson and Chien, 2002). Retinal projections were visualized at 3dpf in DNGα/Citrine, DNβγ/Citrine, or Citrine expressing embryos (Figure 1C,D). We examined the frequency of ectopic misprojections. We also examined retinal projections from individual eyes by the orthograde transport of the lipophilic fluorescent tracers DiI or DiD injected into the eye at 5dpf (Figure 1E,F). This approach revealed many of the same errors that are apparent in the 3dpf preparations but could also detect

ipsilateral misprojections to the incorrect tectum. Retinal projections were examined in transgenic lines from two independent founders for each dominant negative construct.

The gross morphology of the eye is normal in *Ath7*:Gal4 and dominant negative Gα or βγ expressing embryos. RGC axons and some additional retinal cell types including photoreceptor neurons are fluorescently labeled (Figure 1C). No errors are detected in the exit of retinal axons from the eye in any condition. No significant errors are observed in embryos expressing DNGα_i. Occasional ectopic anterior misprojections are observed in DNG$\alpha_{q;}$ but not in DNGα_S, DNGα_q, or DNG$\beta\gamma$ expressing embryos (Figure 1G). More strikingly, we observe ipsilateral misprojections of RGC axons expressing either DNG$\beta\gamma$ or DNGα_S (Fig 1D,F,H). Embryos containing multiple copies of the DNGα_S transgene have an increased penetrance of the ipsilateral misprojection phenotype (Fig1H). Injection of capped mRNA encoding a constitutively active form of Gα_S, caGα_S (Q227L), rescues ipsilateral retinal misprojections of embryos expressing the DNGα_S transgene (Figure 1I).

Gα_S is transcribed from the imprinted GNAS locus and long and short isoforms are expressed in temporally distinct patterns throughout the embryo (Krechowec et al., 2012). These isoforms vary at their N-termini and are indistinguishable in their ability to activate adenylyl cyclases (Novotny and Svoboda, 1998). The DNGα_S targets both isoforms. To confirm expression of Gα_S in the developing zebrafish retina, we performed *in situ* hybridization using a C-terminal directed probe that recognizes both long and short isoforms. We detect broad expression of Gα_S as reported previously in other organisms (Li et al., 2000). Gα_S is expressed within the RGC layer at the time retinal axons cross the midline (data not shown), consistent with previous RT-PCR based expression studies in the adult zebrafish (Oka and Korsching, 2011). Our findings show

that if $G\alpha_S$ activity is reduced, then abnormal ipsilateral projections form, and these errors are corrected if constitutive $G\alpha_S$ activity is expressed in retinal axons. We conclude that $G\alpha_S$ is required within RGCs for retinal axons to cross normally.

Retinal misprojections in DN$G\alpha_S$ transgenics phenocopy *adcy8*, *sema3D*, *sema3E*, or *nrp1a* knockdown.

We recently described a role for the calcium-calmodulin adenylyl cyclases Adcy1b and Adcy8 in promoting retinal midline crossing (Xu et al., 2010). Knockdown of *adcy1b* or *adcy8* produce ipsilateral retinal misprojections similar to those seen in *Ath7* promoter driven DN$G\alpha_S$ transgenics (Figure 2 A,B; Xu et al., 2010). Ipsilateral retinal misprojections can also be induced by knocking down members of the class 3 semaphorin family of axon guidance cues or one of their key receptor components. In mouse, the semaphorin receptor component Neuropilin1 (Nrp1) was recently shown to promote midline crossing in the optic chiasm in a VEGF dependent manner (Erskine et al., 2011). Sema6D, NRCAM, and PlexinA1 expressed at the ventral midline also promote retinal midline crossing (Kuwajima et al., 2012). In zebrafish, knockdown of *sema3D* induces ipsilateral misprojections of retinal axons (Sakai and Halloran, 2006). Here we extend these findings to report that *nrp1a*, *sema3D*, and *sema3E* all cooperate to promote retinal midline crossing in zebrafish embryos. Morpholino induced knockdown of *nrp1a*, *sema3D*, or *sema3E* each produce ipsilateral misprojections similar to those observed in *Ath7* promoter driven DN$G\alpha_S$ transgenics (Figure 2 A,C,D,E). These findings were replicated with two independent morpholinos for each target and all produced ipsilateral projections (Fig 2G). To further confirm this phenotype, we examined *sema3D*^{sa1661} mutant lines and found that these embryos also have ipsilateral retinal misprojections (Figure 2F,G). The somewhat lower penetrance of

the ipsilateral misprojection phenotype was surprising as this mutant line contains a premature stop codon in the sema domain and is expected to be a null. One possible explanation is that general toxicity or off target effects of the morpholinos sensitize the fish for *sema3D* specific knockdown. Consistent with this idea, we observe occasional (~4%) ipsilateral projections in embryos injected with a random control morpholino. Ipsilateral misprojections were obtained when any of the semaphorin or neuropilin-directed morpholinos were combined with a morpholino that targets p53 which reduces off target morpholino effects (Robu et al., 2007). Another possibility is that there is a maternal contribution of *sema3D* RNA that is knocked down by the morpholinos we used.

To test whether *sema3D*, *sema3E,* and *nrp1a* work cooperatively, we performed pairwise injections with low doses of morpholinos to these targets. Morpholinos against *sema3D* and *nrp1a*, *sema3E* and *nrp1a*, or *sema3D* and *sema3E* all act synergistically; inducing more ipsilateral misprojections than predicted by summing the effects of each morpholino alone (Figure 2H). In contrast, morpholinos targeting *sema3A1* do not synergize with morpholinos to *nrp1a*. From these findings we conclude that cAMP signaling through $G\alpha_S$ and Adcy8, or Sema3D and Sema3E signaling via Nrp1a, are all required to ensure that retinal axons reliably cross the ventral midline.

Sema3D and _sema3E_ are expressed near the chiasm as RGC axons cross the midline.

After exiting the eye at about 32hpf, the first retinal axons extend towards and cross the ventral midline to form the chiasm between 34-36hpf (Poulain et al., 2010). We carried out *in situ* hybridization on 36hpf embryos to determine whether *sema3D* and *sema3E*

are expressed along the optic tract as retinal axons navigate through the ventral midline. *Sema3D* is expressed at the midline just dorsal and ventral to the chiasm (Figure 3A,C). *Sema3E* is expressed more broadly ventral to the chiasm (Figure 3B,C). Retinal axons traverse the chiasm immediately adjacent to ventrally expressed *sema3D* and *sema3E*, but do not extend axons within the expressing tissues. Within the retina, low levels of *nrp1a*, a co-receptor for class 3 semaphorins, is expressed in 36hpf RGCs (Fig 3D). The expression of *nrp1a* in RGCs is consistent with previous studies that reported low levels of *nrp1b*, *nrp2a*, and *nrp2b* expression in RGCs (Bovenkamp et al., 2004; Liu et al., 2004; Yu et al., 2004). These results demonstrate that *sema3D* and *sema3E* are expressed at the appropriate time and place to help guide retinal axons across the ventral midline.

Ipsilateral misprojections of retinal axons in *nrp1a* morphant embryos can be corrected by re-expression of *nrp1a* in RGCs

Morpholino knockdowns have the potential for off target or non-specific toxic effects that can cloud their interpretation. We therefore used a rescue-based approach to determine whether *nrp1a* is required cell autonomously in RGCs for normal retinal pathfinding. Single eyes in *nrp1a* morphant embryos were electroporated at 24 hpf, well before retinal axons cross the midline, with either plasmids encoding HuC:Gal4 or Ath7:Gal4 to drive UAS expression in RGCs and either a UAS:Gap43-Citrine (control) construct or a UAS:*Nrp1a*;UAS:Gap-43-Citrine (rescue) construct (Figure 4A,B). The axon trajectories of Citrine-labeled RGCs were traced at 4dpf (Figure 4C). A majority of morphant embryos that expressed the control construct were observed to contain ipsilateral retinal misprojections (Figure 4D,F). The frequency of ipsilateral misprojections was significantly reduced in morphants expressing the *nrp1a* rescue construct (Figure 4E,F).

We conclude that *nrp1a* is required within RGCs to facilitate retinal axon crossing at the ventral midline.

Interactions between cAMP and Nrp1a mediated signaling pathways.

Either the expression of a DNGα_S in RGCs or the knockdown of calcium-calmodulin adenylate cyclases induce ipsilateral misprojections of retinal axons. These results suggest that lowered cAMP levels lead to aberrant retinal pathfinding. If this is the case, interfering with Gα_S and calmodulin mediated pathways at the same time should have a synergistic effect in producing retinal pathfinding errors. Generally consistent with this expectation, low doses of an *adcy8* morpholino injected into embryos with a single copy of the DNGα_S transgene induces a higher, although not quite statistically significant, frequency of embryos with ipsilateral retinal misprojections than would be expected by simply summing together the effects of the DNGα_S and the *adcy8* morpholino (Figure 5A). This result raises the possibility that the Gα_S and *adcy8* signaling pathways converge together, very likely at the level of cAMP production.

Since interfering with the activation of adenylate cyclases phenocopies the knockdown of semaphorin signaling components, we hypothesized that cAMP levels regulate semaphorin signaling in retinal axons. To further explore interactions between signaling elements in the cAMP pathway and the semaphorin signaling pathway, we tested whether knockdowns in these two pathways synergize. Low doses of a *nrp1a* morpholino injected into embryos with a single copy of the DNGα_S transgene induces a significantly higher frequency of embryos with ipsilateral retinal misprojections than would be expected by summing together the effects of either manipulation alone (Figure 5B). Even stronger synergistic effects were seen when embryos were injected with

nrp1a and *adcy8* morpholinos (Figure 5C). We conclude that cAMP and Semaphorin/Neuropilin signaling pathways interact to facilitate retinal midline crossing.

Nrp1a transcript levels are decreased in DNGα_S transgenics and in _adcy8_ morphants.

This interaction led us to hypothesize that a cAMP mediated signaling pathway promotes the expression of *nrp1* in RGCs. Imai et al (2006) have proposed that a Gα_S mediated cAMP, PKA, and CREB dependent pathway promotes the expression of *nrp1* in olfactory sensory neurons and thereby helps target their axons along the anterior-posterior axis of the mouse olfactory bulb. (Imai et al., 2006). Adenylyl cyclase 3 (ADCY3) is a critical mediator of cAMP signaling downstream of olfactory receptors. ADCY3 knockout mice are anosmic and display disorganized and mistargeted axonal projections (Wong et al., 2000; Chesler et al., 2007). In vascular progenitors, Nrp1 is upregulated by cAMP through a PKA dependent mechanism (Yamamizu et al., 2009). Based on these findings and our interaction data, we hypothesized that decreased cAMP levels in DNGα_S transgenic or *adcy8* morphant embryos might result in decreased expression of *nrp1a*. We used quantitative PCR (q-PCR) to examine *nrp1a* expression levels in control embryos as compared to DNGα_S expressing or *adcy8* morphant embryos. As both Gα_S and *nrp1a* are broadly expressed in the nervous system, the s1101t:Gal4 line was used to drive DNGα_S expression pan-neuronally in embryos harboring up to three copies of the UAS:DNGα_S transgene (Li et al., 2000; Yu et al., 2004; Oka and Korsching, 2011 Scott and Baier, 2009). First, we carried out q-PCR on genomic DNA from tails of fluorescent embryos to determine the number of DNGα_S copies for each individual embryo. The heads of these animals were then pooled according to copy number. RNA extracted from pooled groups was reverse transcribed to cDNA. *Nrp1a* expression levels

were determined for each pool and normalized to levels of non-fluorescent siblings. Pools containing one copy of UAS:DNGα_S have small but significantly reduced levels of *nrp1a* expression, while expression of more than one UAS:dnGα_S copy reduces *nrp1a* expression levels dramatically (Figure 6A). Similar results were observed for *nrp1b* expression (data not shown). *Nrp1a* expression is also significantly reduced in wild type embryos injected with *adcy8* morpholinos as compared to uninjected age-matched controls (Figure 6B). There is no significant change in another cell surface receptor, *cxcr4*, in either DNGα_S expressing or in *adcy8* morphant embryos (Figure 6C,D). These results show that interfering with the production of cAMP through either Gα_S or Adcy8 mediated pathways induces a reduction in *nrp1a* expression, consistent with the hypothesis that cAMP signaling affects retinal axon midline crossing through the regulation of *nrp1* expression.

Expressing *nrp1a* in RGCs rescues retinal misprojections in *adcy8* morphants

We hypothesized that reduced cAMP causes a reduction in *nrp1a* levels in RGCs, and that as a consequence, retinal axons misproject ipsilaterally because they cannot respond appropriately to *sema3D* and *sema3E* at the ventral midline. If true, re-expressing *nrp1a* within cAMP deficient RGCs should reestablish more normal retinal crossing in *adcy8* morphant embryos. Control or morphant embryos containing the *Ath7*:Gal4 transgene and either the UAS:Gap43-Citrine (control) or UAS:*nrp1a*;UAS:Gap43-Citrine (rescue) transgenes were allowed to develop to 3 dpf (Figure 7A). One eye was removed and the larvae allowed to develop further to 5 dpf (Figure 7B,C). The optic nerve and tract from the damaged eye degenerates during the two-day period after eye removal and the projection from the remaining eye is visualized by the expression of Gap43-Citrine (Figure 7D). In non-morphant embryos expressing a

control construct, a small proportion of embryos have barely discernable ipsilateral projections. These may represent axons that have not completely degenerated, misrouted newly extended retinal axons from the remaining eye, or sprouting from the intact retinal tract. Previous studies in *Xenopus* detected aberrant ipsilateral connections between the intact eye and the ipsilateral tectum after relatively long survival times when one eye was removed (Fraser and Hunt, 1980). Roughly the same proportion of embryos in all conditions displayed these very weak ipsilateral projections. Consistent with our previous observations, retinal axons in nearly half of *adcy8* morphant embryos had strong ipsilateral misprojections (Fig 7E,F). Expression of the *nrp1a* rescue construct in *adcy8* morphants reduced strong misprojections to control levels (Fig 7F). Overexpression of *nrp1a* in non-morphant embryos did not have any observable effect. We conclude that retinal ipsilateral pathfinding errors observed in *adcy8* morphants can be explained by reduced *nrp1* expression levels.

DISCUSSION

We undertook this study to explore the possibility that GPCR-mediated signaling helps guide axons in the developing nervous system. We previously showed that activation of the G-protein coupled receptors CXCR4 or mGluR1 reduce axonal responses to repellent cues through a $G\alpha_i$, $G\alpha_q$, $G\beta\gamma$, and cAMP mediated signaling pathway. (Twery and Raper, 2011). This effect is rapid, occurring over a timescale of minutes, and is therefore likely to involve a signaling process that is localized to the growth cone. In contrast, in this study we find that a presumed elevation of cAMP mediated through $G\alpha_S$ and/or Adcy8 induces elevated mRNA levels for a key axonal guidance receptor component, Neuropilin1 (Nrp1). This may represent a slower, countervailing influence on guidance receptor activity that is complementary to our previous findings.

The regulation of guidance receptor expression or availability influences axon guidance in a wide variety of systems (Huber et al., 2003; O'Donnell et al., 2009). For example, ERB/Neuregulin1 signaling regulates surface levels of Nrp1 in DRG axons through an unknown mechanism (Hancock et al., 2011). Consequently, the axon trajectories of TrkA expressing DRG sensory axons in the spinal cord of neuregulin-1 mutants resemble those of Sema3A null animals. The semaphorin receptor PlexinA1 is upregulated in spinal commissural axons upon contact with the floorplate, making them responsive to sema3B expressed at the midline, and thereby promoting a repellent interaction that drives them contralaterally (Zou et al., 2000; Nawabi et al., 2010). Recent work suggests that local GDNF signaling from the floorplate increases PlexinA1 on commissural axon growth cones by inhibiting calpain-mediated degradation of the receptor (Charoy et al., 2012). Similarly, Xenopus RGCs are repelled by Sema3A only after crossing the midline (Campbell et al., 2001). Cyclic nucleotide levels can control the surface levels of key guidance receptors. The sensitivity of pre-crossing commissural axons to netrin is enhanced by an adenylyl cyclase and cAMP-dependent PKA activity that promotes insertion of DCC into the cell membrane (Bouchard et al., 2004; Moore and Kennedy, 2006).

We do not know the mechanism by which cAMP induces elevated *nrp1a* levels in RGCs. Either the transcription or stability of *nrp1a* mRNA could be affected. One appealing hypothesis is that cAMP signaling induces *nrp1a* transcription through its canonical effector, the bZIP transcription factor CREB. GPCR activation can lead to elevated cAMP, the phosphorylation of PKA, and the resulting activation of CREB (Lonze and Ginty, 2002; Carlezon et al., 2005). In mouse, Nrp1 expression increases in olfactory

sensory neurons expressing constitutively active GαS, PKA, or CREB; suggesting that cAMP acts via CREB to promote Nrp1 expression (Imai et al., 2006). CREB knockout mice display axon guidance errors independent of neurotrophin-dependent survival defects (Lonze et al., 2002). NGF maintains neuropilin expression in sensory axons, another hint that CREB might mediate the defects we observe in DNGα_S transgenics and *adcy8* morphants (Pond et al., 2002). Similar but not identical axon guidance and branching errors are observed in mouse CREB as compared to Nrp1$^{sema-/-}$ mutants, perhaps because CREB affects additional signaling pathways besides those that are Nrp1 dependent (Rudolph et al., 1998; Lonze et al., 2002; Gu et al., 2003). There are multiple consensus binding sites for CREB within the 5kb of sequence upstream of zebrafish *nrp1a* and *nrp1b*. This is consistent with the idea that CREB could mediate cAMP dependent *nrp1a* expression. Alternatively, direct up-regulation of transcription could occur via another cAMP dependent bZIP transcription factor such as CREM or ATF; or through a PKA independent mediator such as EPAC (Sands and Palmer, 2008).

Our results suggest that GPCR activation induces sufficient Neuropilin1 expression for normal retinal axon pathfinding. A major outstanding question is which Gα_S coupled GPCRs are responsible for maintaining cAMP levels in RGCs? The Gα_S coupled A2b receptor maintains cAMP levels in young Xenopus RGCs and thereby promotes their attraction to netrin at the optic nerve head (Shewan et al., 2002). A2b is also expressed in zebrafish retinae (Boehmler et al., 2009). D1 type dopamine receptors, which canonically induce cAMP production, have been shown to affect neurite outgrowth in chick retinal explants and are expressed in zebrafish RGCs (Lankford et al., 1988; Mora-Ferrer et al., 1999). Other GPCRs that are not thought to act through Gα_S may elevate cAMP levels through alternate pathways (Daaka et al., 1997; Selkirk et al., 2001). Cxcr4

is expressed in RGCs and its activation has been proposed to elevate cAMP levels (Xu et al., 2010). Cxcr4 is required for retinal axons to project normally out of the eye and its activation can affect retinal axon responses to the midline retinal repellent Slit2 (Li et al., 2005; Chalasani et al., 2007). Sonic Hedgehog signals via the 7TM GPCR Smoothened which is expressed in RGCs. Its loss induces RGC misprojections in mouse, Xenopus, and zebrafish (Barres et al., 2005; Sánchez-Camacho and Bovolenta, 2008; Fabre et al., 2010; Gordon et al., 2010). Some or all of these misprojections can be attributed to the important role Shh plays in the specification of midline structures (Barres et al., 2005). We previously showed that mGluRs can modulate axonal responses to repellents, presumably through an elevation of cAMP. Zebrafish homologues of mGlur1, mGlur5, and mGlur6 are all expressed In the retina (data not shown). Potentially, any or all of these and other GPCRs expressed in RGCs could help maintain an elevated level of cAMP that is consistent with normal retinal axon crossing at the ventral midline.

Sema3D has been reported to promote retinal axon crossing at the optic chiasm (Sakai and Halloran, 2006). We demonstrate that Sema3D and Sema3E are both expressed immediately adjacent to the zebrafish optic chiasm and that they facilitate retinal axon crossing through their interaction with Neuropilin1 expressed in RGCs. The simplest explanation for our results is that Sema3D and Sema3E act as permissive factors or attractants that facilitate retinal axon growth across the midline. At the mouse optic chiasm, Sema6D, PlexinA1, and NRCAM are all expressed on midline glial cells and together facilitate the crossing of RGC axons (Kuwajima et al., 2012). Retinal axons misproject ipsilaterally in Neuropilin-1 mutant mice, although its ligand is VEGF expressed near the midline rather than a semaphorin (Erskine et al., 2011). Our results are consistent with Sema3D and Sema3E promoting retinal axon outgrowth. In other

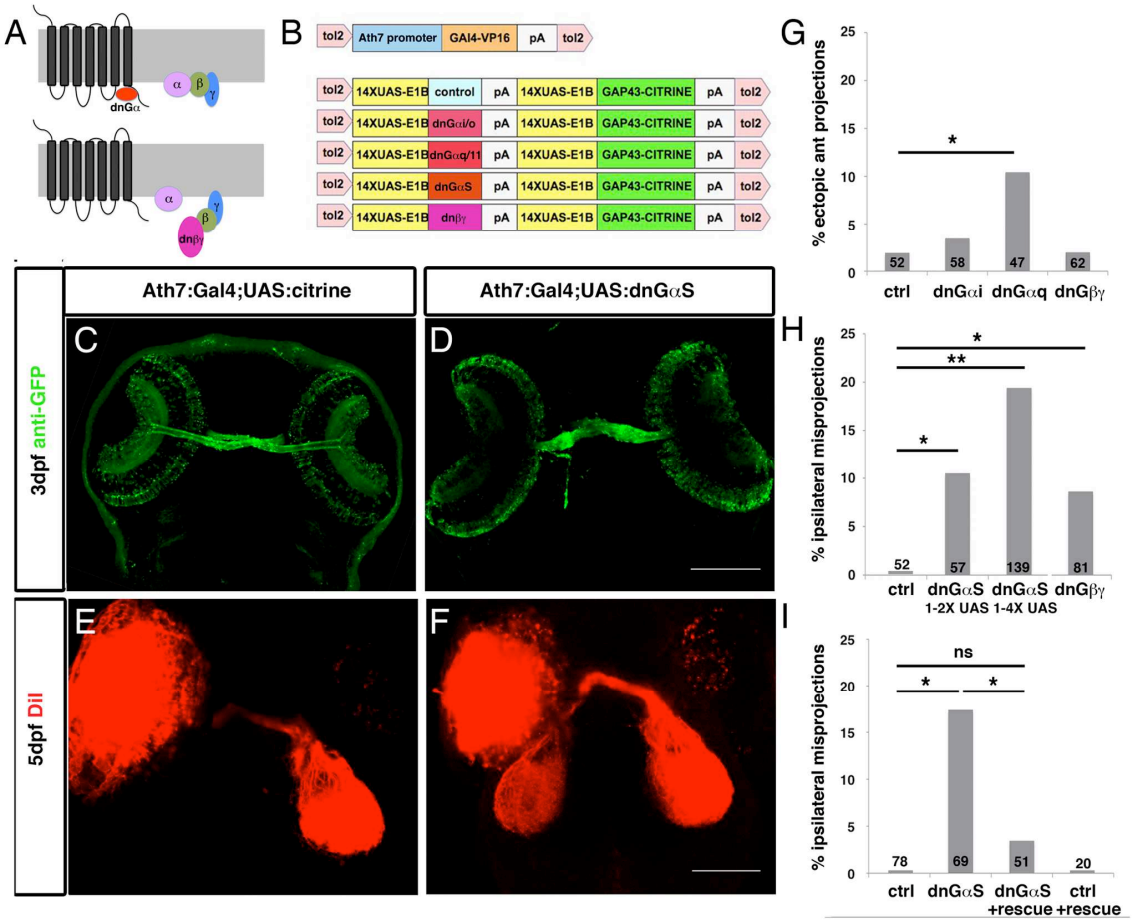
systems, each of these semaphorins can act as either a repellent or an attractant depending upon the receptor components expressed in responding axons (Wolman et al., 2004; Chauvet et al., 2007). Sema3D is reported to promote the crossing of axons in the anterior commissure but to repel ventral RGC axons in the zebrafish tectum (Liu et al., 2004; Wolman et al., 2004). Similarly, Sema3E is reported to act as an attractant for subiculo-mammilary neurons through Nrp1, but as repellent for corticospinal axons through a Nrp1-independent mechanism (Chauvet et al., 2007).

The finding that Sema3D or Sema3E can act as repellents in other systems, and the reported finding that Sema3D excludes dorsal retinal axons from the ventral tectum (Liu et al., 2004), raise the possibility that Sema3D and Sema3E are not attractants for retinal axons at the chiasm. They may instead act as repellents that either drive crossing retinal axons into a more permissive pathway on the dorsoventral axis, or repel retinal axons that have already reached the midline over to the contralateral side. Repellents have been proposed to channel axons into more permissive pathways. For example, motor axons innervating dorsal limbs are driven away from ventral limb territories by ventrally expressed Ephrin-As and their growth is promoted by dorsally expressed EphAs (Marquardt et al., 2005; Bonanomi and Pfaff, 2010). There is also a compelling precedent for semaphorins acting as repellents that drive axons from the midline to the contralateral side. Spinal commissural axons become sensitive to Sema3B only after crossing the floorplate, where GDNF provides a local signal that prevents calpain mediated degradation of the PlexinA1 (Zou et al., 2000; Charoy et al., 2012). Using a combination of axon outgrowth experiments *in vitro* and misexpression experiments *in vivo*, we have been unable to conclusively determine whether retinal axons are attracted or repelled by Sema3D or Sema3E. However, we observe axons extending towards and

into patches of cells misexpressing Sema3D or Sema3E near the chiasm, arguing against a repulsive role for either one of them at the midline (data not shown).

These studies were motivated by the hypothesis that GPCRs act as axonal guidance receptors *in vivo*. Instead, in the retinotectal projection at least, our findings demonstrate an indirect effect of G protein coupled signaling upon the regulation of the canonical axonal guidance receptor, Neuropilin1. We conclude that elevated levels of cAMP induce elevated levels of Nrp1a in RGCs, that Nrp1a is required for retinal axons to interact with Sema3D and Sema3E expressed at the midline, and that this interaction promotes retinal midline crossing. We propose that cAMP levels thereby provide a mechanism for fine-tuning axonal response to repellents over longer time scales corresponding to the transcription and translation of receptors. We further propose that activation of GPCRs or other signaling pathways that control cAMP levels have the more general ability to regulate the expression of axonal guidance receptors. This suggests that as growth cones advance, they can respond to localized signals that regulate cAMP levels, and are thereby reprogrammed to respond differently to the next guidance cues they encounter in their environment.

Figure 3.1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β
γ
dnβγ
B
tol2
Ath7 promoter
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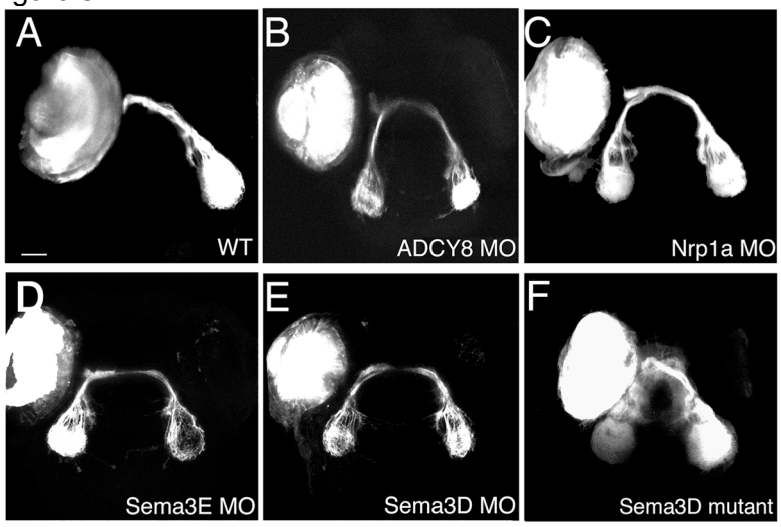
dnGαq/11
dnGαS
dnβγ
GAP43-CITRINE
Ath7:Gal4;UAS:citrine
Ath7:Gal4;UAS:dnGαS
3dpf anti-GFP
5dpf DiI
C
D
E
F
G
% ectopic ant projections
ctrl
dnGαi
dnGαq
dnGβγ
H
% ipsilateral misprojections
dnGαS
1-2X UAS 1-4X UAS
I
ns
+rescue

Figure 3.1. Retinal axons expressing $G\alpha_S$ misproject to the ipsilateral tectum.

We generated multiple transgenic lines expressing dominant negative G-protein signaling reagents and expressed them in retinal ganglion cells (RGCs). ***A,*** The reagents either block G-protein coupled signaling by α-specific steric hindrance of trimeric G-protein binding to activated GPCRs, or by sequestering active subunits. ***B***, Summary of constructs: the *ath*7 promoter drives expression of Gal4 in RGCs; UAS constructs drive both an axonal marker and dominant negative reagents. ***C,E***, RGC axons exit the eye and cross at the midline at the chiasm before projecting contralaterally to their synaptic targets in control embryos. ***D,*** An ectopic ipsilateral misprojection of retinal axons visualized by citrine expression in a $DNG\alpha_S$ transgenic embryo. ***E,*** Filling one eye with lipophilic dye labels retinal axons extending within the optic tracts to both the ipsi- and contralateral tecta of a $DNG\alpha_S$ transgenic embryo. ***G***, Quantification of ectopic anterior misprojections in control, $DNG\alpha_i$, $DNG\alpha_q$, and $DNG\beta\gamma$ expressing RGCs. ***H***, There is an increase in ipsilateral retinal misprojections with increased expression of $DNG\alpha_S$. $DNG\beta\gamma$ also induces significant ipsilateral misprojections. ***I***, A synthetic mRNA for a constitutively active $G\alpha_S$ rescues ipsilateral misprojections in $DNG\alpha_S$ expressing transgenic embryos. Quantification of ipsilateral misprojections in transgenics expressing $DNG\alpha_S$ or sibling controls not expressing $DNG\alpha_S$ with or without the G_S rescue construct. *=$p<0.05$, **=$p<0.01$ using Fisher's Exact Test. Scale bars, 100υm

Figure 3.2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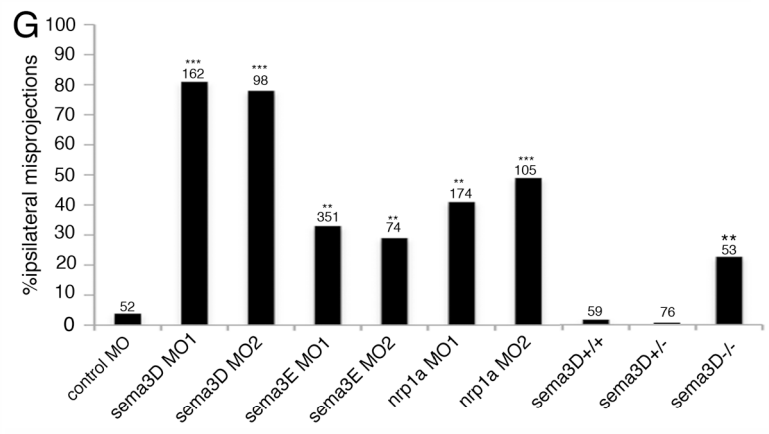
WT
B
ADCY8 MO
C
Nrp1a MO
D
Sema3E MO
E
Sema3D MO
F
Sema3D mutant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100
90
80
70
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10
0
52
162
98
351
74
174
105
59
76
53
control MO
sema3D MO1
sema3D MO2
sema3E MO1
sema3E MO2
nrp1a MO1
nrp1a MO2
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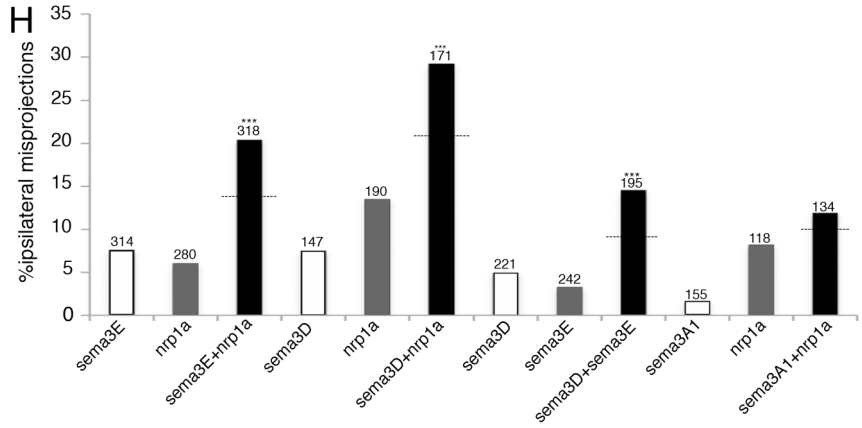
sema3D+/-
sema3D-/-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20
15
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314
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318
147
190
171
221
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155
118
134
sema3E
nrp1a
sema3E+nrp1a
sema3D
nrp1a
sema3D+nrp1a
sema3D
sema3E
sema3D+sema3E
sema3A1
nrp1a
sema3A1+nrp1a

Figure 3.2. Expression of DNGα_S in retinal ganglion cells phenocopies knockdown of *adcy8* or semaphorin signaling components.

A, All retinal axons cross the midline and project to the contralateral tectum in 5dpf WT embryos. Ipsilateral misprojections are observed in ***C,*** *Nrp1a* morphant, ***E,*** *Sema3D* morphant, and ***F,*** *Sema3D* mutant embryos, and ***D,*** *Sema3E* morphant. Ipsilateral misprojections are also observed in ***B,*** *Adcy8* morphant embryos. ***G***, Ipsilateral misprojections are observed using either of two independent morpholinos for *nrp1a*, *sema3D*, or *sema3E* and in mutant embryos. **, p=<0.001, ***p,=<0.0001 using the Fisher exact test. Sema and Nrp1 morphant embryos were compared to embryos injected with control morpholino. Sema3D mutants were compared to WT sibling controls. ***H***, Co-injection of low doses of pairs of morpholinos targeting *nrp1a*, *sema3d*, or *sema3E* produce synergistic increases in ipsilateral misprojections. The proportions of ipsilateral retinal misprojections induced by knocking down each combination of Class 3 semaphorin and/or Nrp1a are indicated. The white and gray bars show responses to half doses of morpholino targeting class 3 semaphorins and Nrp1a respectively. The black bars indicate the response to a combination of half doses of morpholinos. The number of eyes examined in each condition is indicated above each column. Stars above the columns indicate that the percentage of ipsilateral misprojections of retinal axons induced by the combination of half doses is significantly higher than would be expected by assuming that the effects of half doses add together independently ***: p=<.00001 using a Monte Carlo based analysis that estimates the probability that observed phenotypes are independent and additive. Scale bars in A-F, 50υm

Figure 3.3

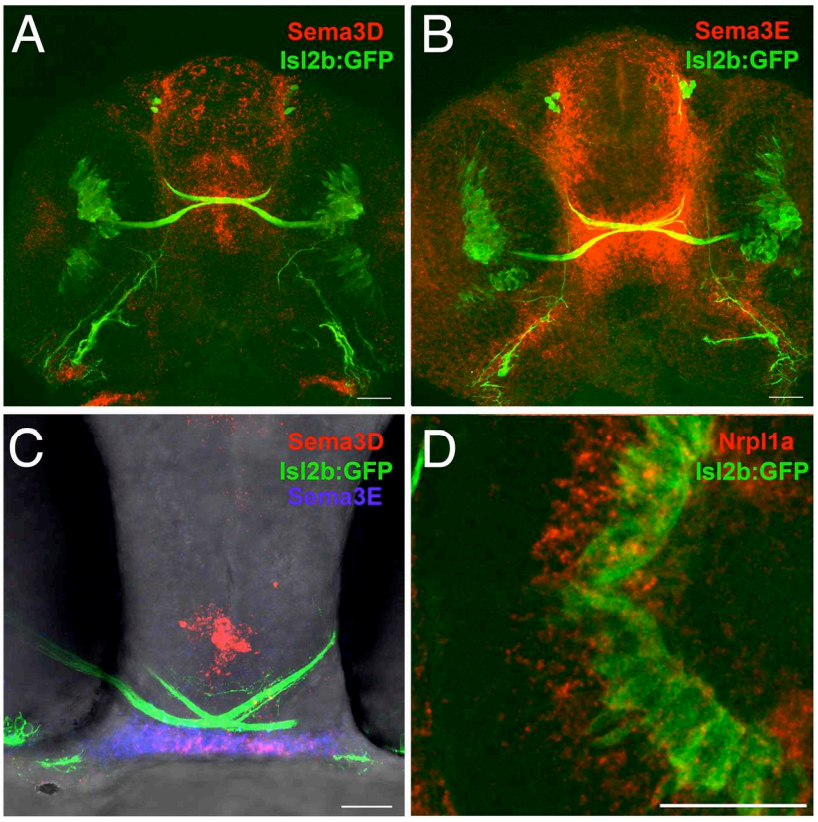

A
Sema3D
Isl2b:GFP
B
Sema3E
Isl2b:GFP
C
Sema3D
Isl2b:GFP
Sema3E
D
Nrpl1a
Isl2b:GFP

Figure 3.3. *Sema3D* and *sema3E* are expressed near the chiasm and *nrp1A* is expressed in retinal ganglion cells.

A subset of retinal ganglion cells and their axonal projections were visualized with *Isl2b*:GFP transgene expression and the expression patterns of semaphorin signaling components were detected by *in situ* hybridization with the indicated RNA probes. ***A-B***. Ventral views of 36hpf embryos showing that ***A,*** *Sema3D* and ***B,*** *Sema3E* are expressed in the vicinity of the optic chiasm. ***C,*** A frontal view demonstrates that *Sema3D* (red) is expressed both dorsal and ventral to crossing retinal axons (green), while *Sema3E* (blue) is expressed ventral to the chiasm. ***D,*** *Nrpl1a* (red) is expressed in retinal ganglion cells (green) at 36hpf. Scale bars = 50uM

Figure 3.4

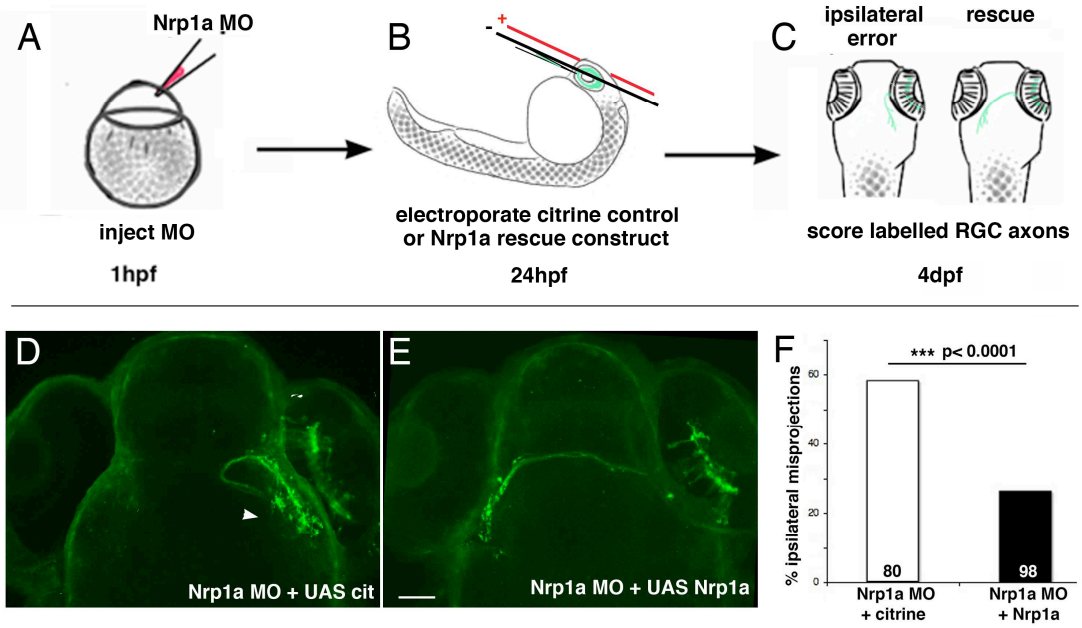

A
Nrp1a MO
inject MO
1hpf
B
-
+
electroporate citrine control
or Nrp1a rescue construct
24hpf
C
ipsilateral
error
rescue
score labelled RGC axons
4dpf
D
Nrp1a MO + UAS cit
E
Nrp1a MO + UAS Nrp1a
F
*** p< 0.0001
% ipsilateral misprojections
0
20
40
60
80
98
Nrp1a MO
+ citrine
Nrp1a MO
+ Nrp1a

Figure 3.4. The expression of *Nrp1a* in retinal ganglion cells rescues ipsilateral misprojections in *Nrp1a* morphants.

A, One-cell stage embryos were injected with *nrp1a* MO and allowed to develop to 24hpf when ***B,*** plasmids containing *Atoh7*:Gal4 and either UAS:Citrine (control condition) or UAS:*nrp1a*:UAS:Citrine (rescue condition) were electroporated into one eye. ***C,*** Citrine labeled retinal projections were visualized at 4dpf. ***D-E,*** Representative images of electroporated embryos. Background staining unrelated to the retinal projections was removed prior to making these maximum projection images (see Methods). ***D,*** An example of ipsilateral misprojecting retinal axons in an *nrp1a* morphant expressing the control construct (arrowhead). ***E,*** An example of correctly routed retinal projections in a *nrp1A* morphant in which the *nrp1a* rescue construct is expressed in the labeled RGCs. ***F,*** Quantification of results represented in ***D,E***. *nrp1a* expression in RGCs significantly reduced the frequency of ipsilateral misprojections in *nrp1a* morphant embryos). The number of zebrafish larvae in each condition are indicated in the columns. $p<0.0001$ by Fisher's Exact Test.

Figure 3.5.

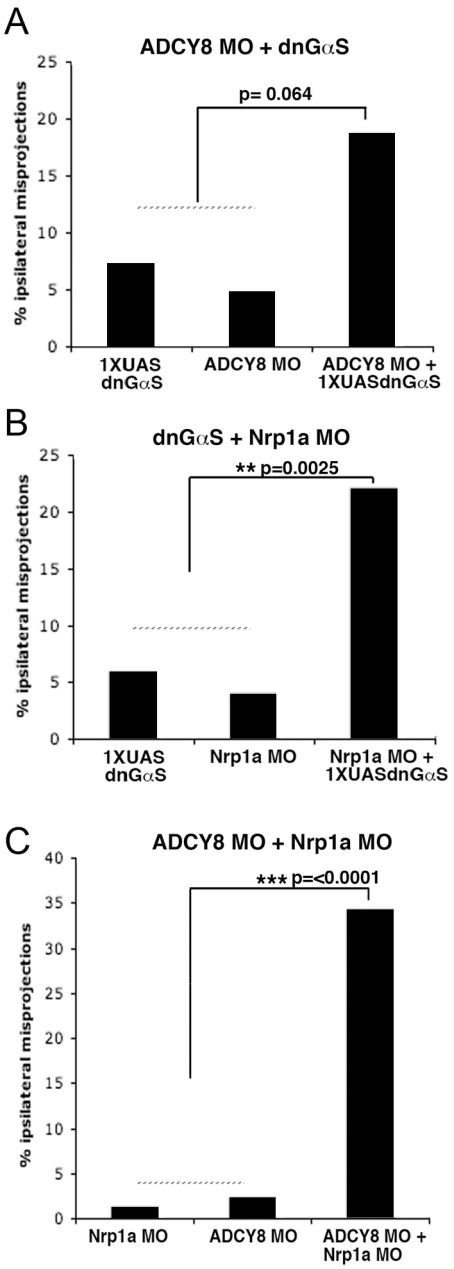

A
ADCY8 MO + dnGαS
p= 0.064
% ipsilateral misprojections
25
20
15
10
5
0
1XUAS dnGαS
ADCY8 MO
ADCY8 MO + 1XUASdnGαS
B
dnGαS + Nrp1a MO
** p=0.0025
% ipsilateral misprojections
25
20
15
10
5
0
1XUAS dnGαS
Nrp1a MO
Nrp1a MO + 1XUASdnGαS
C
ADCY8 MO + Nrp1a MO
*** p=<0.0001
% ipsilateral misprojections
40
35
30
25
20
15
10
5
0
Nrp1a MO
ADCY8 MO
ADCY8 MO + Nrp1a MO

Figure 3.5. Synergistic interactions between cAMP and Nrp1a mediated signaling pathways.

A-C Quantification of ipsilateral misprojections detected by DiI or DiD labeling at 5dpf. **A,** Embryos containing one copy of the UAS:DNGα_S and injected with a low dose of *adcy8* MO have a higher frequency of ipsilateral misprojections than expected if the effects of each perturbation are independent and sum linearly. ***B,*** Similarly, a single copy of UAS:DNGα_S and a low dose of *nrp1a* MO act synergistically to induce ipsilateral misprojections. ***C,*** Co-injection of low doses of *nrp1A* and *adcy8* morpholinos synergize to produce ipsilateral misprojections. In each panel, the dotted line represents the incidence of misprojections expected if the two conditions sum linearly. p values indicate the probability that the combined effect effects are independent and additive.

Figure 3.6

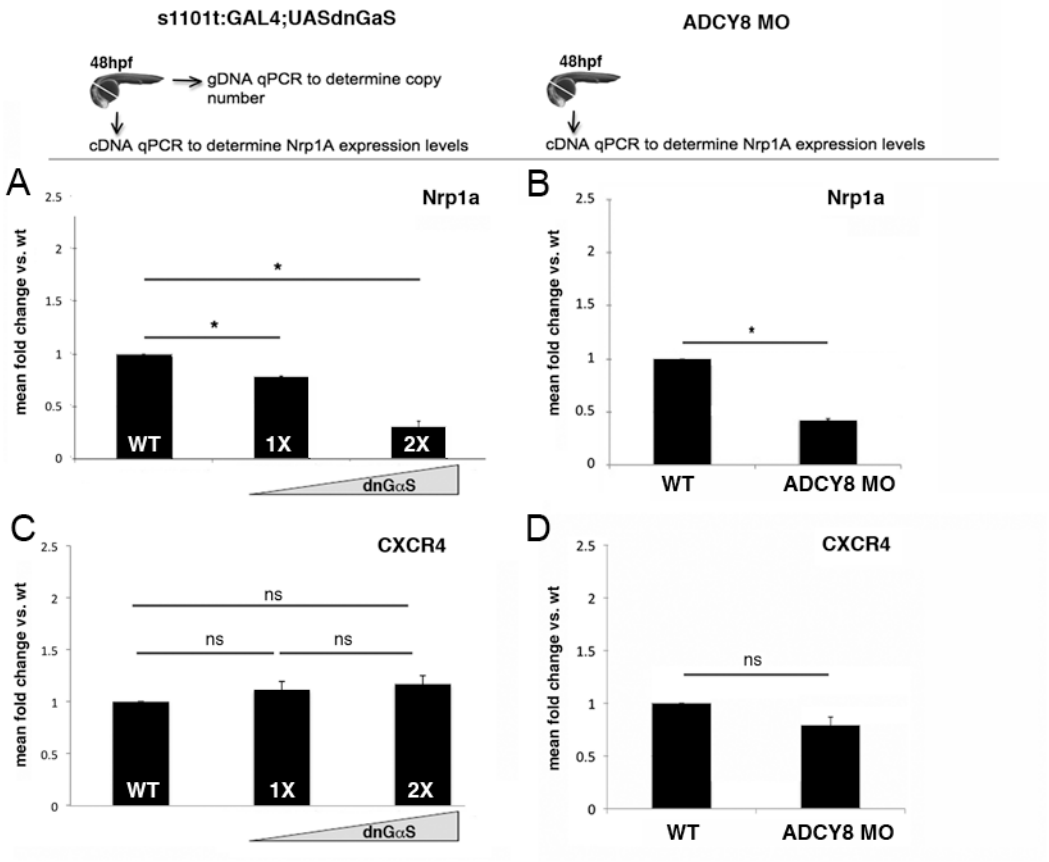

s1101t:GAL4;UASdnGaS
48hpf
gDNA qPCR to determine copy number
cDNA qPCR to determine Nrp1A expression levels
ADCY8 MO
48hpf
cDNA qPCR to determine Nrp1A expression levels
A
Nrp1a
mean fold change vs. wt
WT
1X
2X
dnGαS
B
Nrp1a
WT
ADCY8 MO
C
CXCR4
ns
ns
ns
WT
1X
2X
dnGαS
D
CXCR4
ns
WT
ADCY8 MO

Figure 3.6. *Nrp1a* transcript levels are decreased in DNGα$_S$ transgenic and in *adcy8* morphant embryos.

A, As DNG $_S$ expression is increased, *nrp1a* expression is reduced. qPCR was performed to measure *nrp1a* expression using cDNA prepared from pools of 48hpf heads containing either one or two copies of the DNGα$_S$ transgene. DNGα$_S$ transgene expression was driven by the panneuronally expressed s1101t:Gal4 transgene. cDNA was generated from pooled non-fluorescent siblings to serve as a control. ***B,*** Expression of *nrp1a* was similarly reduced in *adcy8* morphant embryos. ***C,D,*** Expression of *cxcr4* was not affected by either DNG $_S$ expression or *adcy8* knockdown. *=p <0.05, using Student's t-test

Figure 3.7.

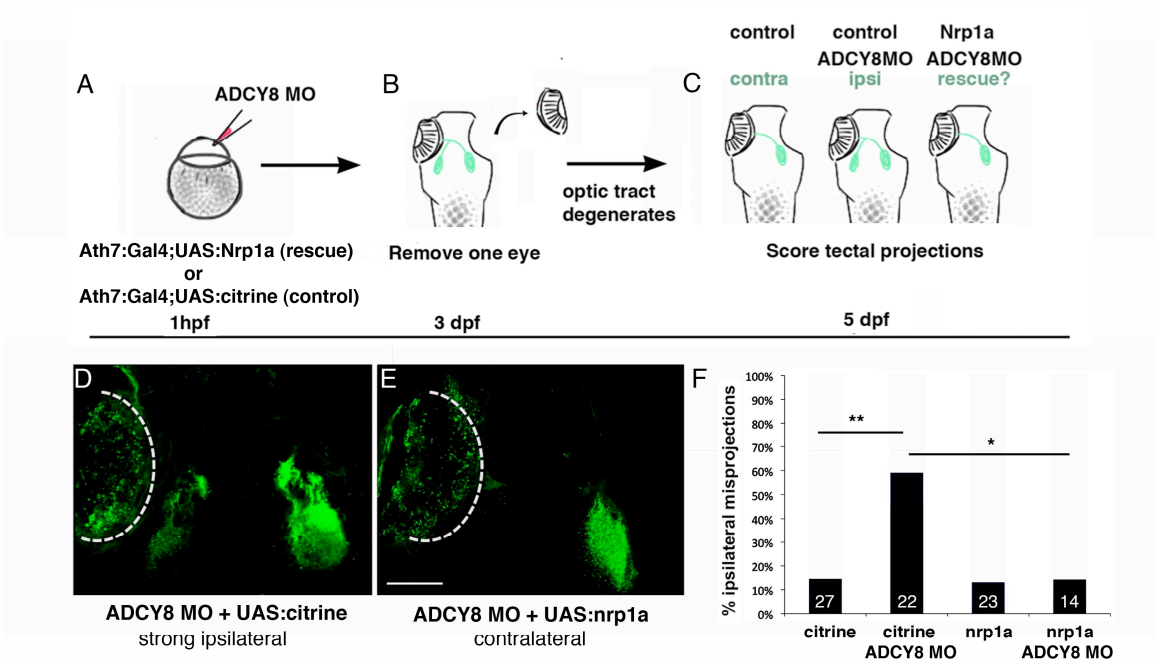

A
ADCY8 MO
B
optic tract degenerates
C
control
control ADCY8MO
Nrp1a ADCY8MO
contra
ipsi
rescue?
Ath7:Gal4;UAS:Nrp1a (rescue)
or
Ath7:Gal4;UAS:citrine (control)
1hpf
Remove one eye
3 dpf
Score tectal projections
5 dpf
D
E
F
ADCY8 MO + UAS:citrine
strong ipsilateral
ADCY8 MO + UAS:nrp1a
contralateral
% ipsilateral misprojections
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
**
*
27
22
23
14
citrine
citrine ADCY8 MO
nrp1a
nrp1a ADCY8 MO

Figure 3.7. Retinal misprojections in *adcy8* morphants are rescued by expressing *nrp1a* in RGCs.

A**, Adcy8* morphant or non-morphant embryos were produced from just-fertilized eggs containing either the *Atoh7*:Gal4; UAS:citrine (control) or the *Atoh7*:Gal4; UAS:*Nrp1A*:UAS:citrine (rescue) transgenes. ***B, One eye was removed from each fluorescent embryo at 3 dpf. The optic tract from the removed eye degenerates over the next two days. ***C,*** Embryos were analyzed for ipsilateral misprojections at 5dpf. ***D**,* Representative image in an *adcy8* morphant embryo in which RGCs expressing the control construct are visualized by their expression of citrine. Retinal axons project to both the contra and ipsliateral tecta. ***E**,* Representative image of a contralateral projection in an *adcy8* morphant embryo in which RGCs express the UAS:nrp1a rescue construct. ***F**,* A comparison of ipsilateral misprojections in RGCs expressing either the control construct or the *nrp1a* rescue construct in *adcy8* morphant embryos. Expression of the rescue construct corrects ipsilateral misprojections. Expression of the rescue construct in non-morphant embryos had no observable affect. *: p<= 0.05; **:<= 0.01 by Fisher's exact test. Scale Bar = 100um

CHAPTER 4: FUTURE DIRECTIONS

The previous chapters describe two means by which GPCR and Ca^{2+} modulation of cAMP levels direct navigating axons in the visual system – both by modulating responses to repellent cues, and by promoting expression of axon guidance receptors. There are a number of open questions including: which receptors contribute to signaling, how cAMP regulates Nrp1 transcription levels and how Nrp1 works to promote midline crossing in retinal axons.

Which GPCRs contribute to cAMP levels in navigating retinal axons?

A major outstanding question is which GPCRs are responsible for maintaining or promoting cAMP levels in navigating RGCs. I took a four-fold approach to identify candidate receptors based on **(1)** distribution of receptors which canonically couple to GαS, **(2)** metabotropic glutamate receptors, which we had previously identified in cAMP mediated modulation of guidance decisions **(3)** gene expression data for GPCRs that are expressed in RGCs during development and **(4)** identifying GPCRs already implicated in cell motility and axon guidance. Since GPCRs can activate multiple G alphas, or promote cAMP signaling via the ®© subunit's PLC mediated upregulation of Ca^{2+} signaling, this broad approach seemed reasonable. (Oldham and Hamm, 2008)

GPCRs that canonically couple to GαS

Candidate GPCRs that are canonically coupled to GαS, include the, serotonin (5-HT) receptors types H4 and H7, the adenosine receptor types A2a and A2b, β-adrenergic receptors types β1 β2 and β3, Dopamine Receptors (D1 type), Calcitonin receptor and histamine H2 receptor.

Adenosine Receptors A2a and A2b

Adenosine2a: receptor expression within the retina is restricted to the vasculature in mammalian cells, and is not detected in RGCs in zebrafish, so it was not considered as a candidate. (Taomoto et al., 2000; Boehmler et al., 2009)

Adenosine2b: The adenosine receptor 2b receptor is expressed in RGCs and has been implicated in axon guidance, both independently, and in a more controversial role as a component of netrin signal transduction. Corset et al (2000) carried out a yeast two hybrid screen to identify binding partners for the netrin receptor DCC and identified a 23 aa fragment matching the intracellular domain of the GαS and GαQ coupled A2b receptor. (Corset et al., 2000) CoIP experiments revealed a netrin-dependent interaction between A2b and DCC and blockade of A2b abrogates netrin mediated attraction of commissural axon explants. (Corset et al., 2000) In contrast, a paper from the Tessier-Lavigne lab used sepharose bead binding assays to demonstrate that the extracellular domain of DCC can bind directly to netrin in the absence of A2b, and further used inhibitors of adenosine 2b in netrin mediated turning assays to demonstrate that DCC mediated attraction occurs irrespective of the status of adenosine receptor signaling. (Stein et al., 2001) Independent from a direct role in transducing attractive netrin signaling, A2b promotes membrane insertion of the repellent receptor Unc-5 through a PKC dependent mechanism.(Williams et al., 2003; McKenna et al., 2008) In the developing retinal projection, A2b maintains cAMP levels in young Xenopus RGCs to promote attraction to netrin at the optic nerve head. (Shewan et al., 2002) In zebrafish, A2b is expressed in the retina, while netrin is expressed at the midline and at the eye exit point. (Shewan et al., 2002; Pittman et al., 2008; Boehmler et al., 2009) In higher

vertebrates netrin has been proposed to play an early role in retinal guidance, attracting axons to the eye exit point at the optic nerve head (Deiner et al., 1997) Though retinal phenotypes have not been reported in zebrafish netrin mutants or morphants, the RGC axon guidance defects of the Pax2a mutant, *no-isthmus* (*noi*), have been partially ascribed to mislocalization of netrin in the optic stalk and at the midline. (Macdonald et al., 1997)

As the A2b receptor is canonically coupled to cAMP through both GαQ and GαS, is expressed in RGCs during axonal navigation, and has previously been implicated in axon guidance, we identified it as a candidate for promoting cAMP signaling during axonal navigation in the retinal system. Zebrafish have 2 adenosine 2a receptors, but only one adenosine 2b receptor. (Boehmler et al., 2009) I designed morpholinos targeting the A2b receptor. Knockdown of A2b produced ipsilateral misprojections in a small proportion of embryos (6.6% of embryos 2/30 embryos p=0.07). We followed these with interaction experiments in the context of dnGαS or dnGβγ transgenics. In our preliminary analysis, we did not observe an increase in the penetrance of ipsilateral misprojections over embryos expressing dnGαS alone. These preliminary experiments were carried out in dnGαS embryos containing multiple copies of the dn transgene. If the increased dosage of dnGαS fully inhibited GαS signaling such that it was equivalent to a null mutation, then the lack of increase in phenotype might indicate that A2b is contributing to the dnGαS phenotype. I will repeat these experiments in animals expressing only one copy of the dnGαS and look for a synergistic interaction.

Beta-Adrenergic Receptors types β1 β2 and β3 : In the mouse olfactory system, axons expressing the same olfactory receptor (OR) converge on a single glomerulus. In receptor chimera experiments to determine the relationship between OR expression and

glomerular targeting, expression of the β2 beta-adrenergic receptor coding from the OR-M71 locus, acted as an OR and extended axons to symmetrical targets - a unique glomerulus anterior and ventral to the M71 glomerulus. (Feinstein et al., 2004) Quantitative autoradiography studies on adult zebrafish (Zikopoulous and Dermon 2005 Ampatzis and Dermon 2010) did not analyze the eye, but did identify expression of beta-adrenergic receptors in the optic tectum, as well as strong expression in the olfactory bulb. (Zikopoulos and Dermon, 2005; Ampatzis and Dermon, 2010; Prokosch et al., 2010) An RT-PCR based study of zebrafish beta-adrenergic receptors revealed that ADrb2a is expressed in the eye, but onset of expression doesn't begin until 5dpf. (Wang et al., 2009) These results suggest that beta-adrenergic receptors might mediate targeting during later stages of development, but are unlikely to be involved in midline crossing.

Calcitonin and Calcitonin Like Receptors: In mouse transgenics expressing LacZ under the control of calcitonin receptor promoter, β-gal staining was detected in spinal cord, retina and the olfactory bulb.(Jagger et al., 2000) Early pharmacological studies in Xenopus revealed defects in cell migration, neural tube closure and intraocular distance in response to rearing in calcitonin, though these effects could be toxicity related. (Burgess, 1982, 1985) Neurons produce a unique transcript – Calcitonin Gene Related Peptide (CGRP) from the Calcitonin locus which shares the first two exons with Calcitonin, as well as a unique CGRP exon and a 3'non coding region. (Rosenfeld et al., 1983) CGRP is expressed in trigeminal and sensory axons as well as olfactory neurons, and is used as a marker for nociceptive DRG axons in the spinal cord, though it seems that their role in axon guidance has not been extensively studied. (Rosenfeld et al., 1983; Gibson et al., 1984; Tang et al., 2007). CGRP signals through the CGRP receptor,

which is also coupled to GαS and CGRP is expressed in the retina, where it promotes survival in a coronary occlusion model of stress. (Yang et al., 2011) Thus, calcitonin and CGRP and their receptors might be intriguing candidates for transducing GαS mediated guidance cues in developing retinal axons.

Dopamine D1 receptors: Dopamine D1 and D2 receptors are expressed in the retinal ganglion cell layer in mouse as well as zebrafish. (Lankford et al., 1988; Wagner et al., 1993; Veruki and Wässle, 1996) cAMP is upregulated in RGCs in response to light stimulus via D1 and D2 dopamine receptors, and signaling through D1 receptors promotes neurite outgrowth in cultured chick retinae. (Lankford et al., 1988; Mora-Ferrer et al., 1999; Ogata et al., 2012) In a recent qRT-PCR based study, forskolin treatment of neuroepithelial cells produced an increase in neuropilin1 expression levels, consistent with our findings. However, this effect was not mimicked by the dopamine D1 receptor agonist dihydrexidine, suggesting that Nrp1 expression levels are not mediated by this receptor. (Jassen et al., 2006) Given these data, D1 receptors might act in later activity-dependent processes in neuronal development, such as axonal arborization and pruning, but are not attractive candidates for GαS mediated upregulation of Nrp1 during midline axonal navigation.

Histamine H2 receptors: Though there was an initial report that histamine increased cAMP levels in chick retina via an H2 dependent mechanism, expression studies in both mouse and zebrafish suggest that the H2 receptor is not expressed in retinal ganglion cells. (Airaksinen and Panula, 1988; Nowak and Sek, 1991; Gastinger et al., 2006; Peitsaro et al., 2007) Thus, the H2 receptor is not a candidate. Our lab previously studied the effects of histamine in anti-repellent signaling, and found that histamine

reduced the effects of Slit on RGCs using the same pathway previously described for SDF/CXCR4. (Kriebich and Raper, unpublished observations) Since the antirepellent pathway is PTX sensitive, these signals are likely be transduced through receptors H3 and H4 which are Gαi coupled.

Serotonin Receptors Htr4 and Htr5: No zebrafish homologue of Htr4 has been yet identified, but there are two Htr5 receptors (Htr5a and Htr5al) in the zebrafish genome. No expression data is currently available for either of these genes. Gene expression profiling detects expression of the 5HTR gene in some samples of adult mouse retina (NCBI GEO datasets D:14944852) In the field of axon guidance, serotonin has been identified as a modulator of netrin signaling in thalamocortical axons through the Gαi/o coupled Htr1b and Htr1d. (Bonnin et al., 2007) This finding is consistent with work from our own lab suggesting that serotonin functions to reduce axonal response to repellents. (Kreibich and Raper, unpublished observations) Local application of seratonin to cultured or severed growth cones of two identified neuronal populations of the snail *Helisoma*, demonstrated that these populations respond differently to inhibitory cues. Seratonin induced collapse and inhibited synaptogensis of neuron 19 growthcones, while neuron 5 was unaffected. (Haydon et al., 1984) Serotonin induces growth cone collapse of cultured *Lymnaea stagnalis* cerebral giant cells, while work in Aplysia linked serotonin levels to expression of cell adhesion molecules required for motor neuron fasciculation. (Peter et al., 1994; Koert et al., 2001). While Htr4 and Htr5 might induce or maintain cAMP signals in the developing visual system – the lack of expression data led us to focus on other candidates.

Metabotropic Glutamate Receptors

We previously showed that Class I metabotropic glutamate receptor mGluR1 reduces repellent signaling in RGCs and DRG through the pertussis toxin, cAMP and PKA dependent anti-repellent pathway. (Kreibich et al., 2004) Class I mGluRs promote outgrowth of dopamanergic striatal neurons in organotypic culture, and neurite extension when overexpressed in a neuroblastoma cell line (Plenz and Kitai, 1998; Mion et al., 2001) These receptors interact with heterotrimeric G-proteins GαI, GαQ and GαS through their second intracellular loops, as well as with Calmodulin, Homer scaffolding proteins and Src-family protein tyrosine kinases. (Hermans and Challiss, 2001; Enz, 2012) The Class I mGluR agonist DHPG activates CREB, Elk-1 and ERK through a CamKIV dependent mechanism. (Choe and Wang, 2001)

We identified class I mGlur homologues: mGlur1a, mGlur1b, mGlur5a and mGlur5b, and carried out ISH to assess their expression in the developing zebrafish retina. I observed RGC expression in mGluR1b and mGluR5a transcript in RGCs, consistent with previous expression data in rat. (Hartveit et al., 1995) Morpholino knockdown of these molecules did not produce errors in eye exit, or errors at the chiasm – as assayed by immunostaining at 2 and 3dpf. I did not assess mGluR1 and mGluR5's effects on by lipophilic tracer such as DiI, so reanalysis of these morphants is certainly in order. I also identified new candidate via the ZFIN database, a then-uncharacterized mGlur homologue, GRMA, based on its strong expression in zebrafish RGCs. I carried out 3' and 5'RACE on GRMAs EST and found it to be most similar to a class III mGluR, mGlur6.

mGlur6 was first identified from a rat retinal cDNA library with a probe derived from mGlur1. This screen produced hits for mGlur1-5 as well as a novel receptor – mGluR6. (Nakajima et al., 1993) Like other class III mGluRs, mGluR6 was initially characterized as cAMP inhibiting. In CHO cells stably expressing mGluR6, application of glutamate or

the class III glutamate agonist AP-4 abrogated forskolin mediated cAMP elevations, and requires GαO (Nakajima et al., 1993; Vardi, 1998; Nawy, 1999; Dhingra et al., 2000; Tian and Kammermeier, 2006) mGlur6 is localized to the dendrites of ON-bipolar cells in primates, and restricted to the outer nuclear layer in mouse retina. (Vardi et al., 2000; Quraishi et al., 2007) Interestingly, single cell RT-PCR based studies detected expression of mGlur6 in rat juvenile or regenerating retinal ganglion cells, though it is not detected in uninjured adult tissue. (Tehrani et al., 2000) I generated morpholinos targeting GRMA and found that GRMA knockdown induces ipsilateral misprojections similar to ADCY8, dnGαS, and dnGβγ morphants. I performed a preliminary interaction experiment by knocking down GRMA in animals expressing dnGαS or dnβγ and found that GRMA knockdown did not produce an increase in dnGαS but did produce an increase in dnGβγ ipsilateral misprojections. We are currently following up these experiments in the context of the ADCY8 morphants. Thus, GRMA might signal through the βγ complex to promote crossing in navigating RGC axons.

Gene expression data for GPCRs that are expressed in RGCs during development

Connie Cepko's lab undertook single cell expression of profiling for different molecularly defined cell subtypes in the mouse retina spanning time points from the onset of neurogenesis (e12.5) to birth (p0). Using NF68, or EfB3 as a marker for RGCs, three methods were used to identify transcripts up-regulated in retinal ganglion cells.

Method 1: Hierarchical Clustering Analysis (152 genes, Table 2): **GABAB receptor 1, Cannabinoid Receptor 1, Opioid Receptor Like-1, Celsr3**, Neuropilin1, PlexinA1 and L1.
Method 2: Pairwise comparisons (232 genes, Table 3): ADAM-10, G-Gamma2,3, AKAP6, L1, **Cannabinoid Receptor 1**, Neuropilin1, CRMP1
Method 3: Manual scan (400 genes, Table 4): ADAM11, ADAM19, ADCY1, CamKII, CamKIV, **Cannabinoid Receptor 1**, L1-like, NCAM, CRMP1, **GABAB receptor 1**,

GGamma2,3, L1, **Latrophilin3**, Neuropilin1, **Opioid Receptor Like 1**, Plexin B1, RGS2, RGS4, Sema3A, Sema6D, Shh,
Method 4: Pairwise using EFb3 as a marker (253 genes table 5): **CELSR3**, GGamma3, Neuropilin1, ADAM10, ADAM19, CRMP1, **Cannabinoid receptor 1**, L1

The single cell expression datasets identified several GPCRs expressed in RGCs during development, including the Cannabinoid Receptor (CNR1), The GABAB receptor (GABAB1), and Opioid Like Receptor 1 (Oprl1/NOP1), as well as two members of the adhesion family of G-protein coupled receptors, CELSR3 (Flamingo) and Latrophilin 3. These were each considered in the context of the literature as potential candidates to mediate axon guidance.

Cannabinoid Receptor 1 (CNR1): was identified in each analysis of the SAGE dataset. This expression data, combined with knockdown analysis in zebrafish suggesting a role for CNR1 in axon guidance made CNR1 an exciting candidate. (Watson et al., 2008) However, our own expression analysis in zebrafish suggests that it is not highly expressed in RGCs, consistent with the findings of Watson et al. CoIP experiments carried out in rat brain lysates demonstrated interactions with GαI/o, but not GαS or GαQ. (Mukhopadhyay et al., 2000) Thus, CNR1 is not an attractive candidate for up-regulation of cAMP during RGC axonal navigation.

GABAB1: Xenopus Spinal Neurons turn away from a gradient of baclofen, a GABAB receptor agonist, through a GαI and PKC mediated signaling pathway. (Xiang et al., 2002) We previously identified a role for GABAB1 receptor in mediating antirepellent signaling in cultured chick retinal and sensory axons. (Pisapia and Raper unpublished observations) Following this finding, Alemji Taku, a graduate student in our lab, undertook extensive exploration of a role for GABAB receptor signaling in retinal axon guidance in the developing zebrafish. The GABAB receptor is expressed in the RGC

layer, and GABA is expressed along the optic tract. (Taku and Raper, unpublished observations) A GABAB1.2 receptor mutant, *humphrey bogart* (*hbog*), was recently identified in a screen for alterned response to nicotine. . (Petzold et al., 2009) However, analysis of this mutant did not reveal any retinal axonal misprojections. (Petzold et al 2009 and Alemji Taku unpublished observations) Although, GABAB1 is reported to signaling via GαI/GαO, one study links GABAB receptor signaling to cAMP accumulation in the olfactory bulb (Olianas and Onali, 1999; Padgett and Slesinger, 2010) Thus, the GABAB receptor mediates guidance decisions in other neuronal populations, but does not function in retinal midline crossing.

OPRL1/NOP: Opioid receptor like one (OPRl1), also known as nociceptin receptor (NOP), is structurally similar to opioid receptors, though it doesn't bind the opioid agonist nalaxone. (Lambert, 2008) Zebrafish OPRL1/NOP has been identified and characterized. (Gonzalez-Nunez et al., 2003) zNOP is highly expressed in brain in the adult zebrafish as assayed by qRT-PCR. (Rivas-Boyero et al., 2011) Nociceptin was recently identified as a BDNF target gene, and found to promote neurite outgrowth in hippocampal culture, consistent with previous overexpression studies in NS20Y cells (Saito et al., 1997; Ring et al., 2006) Cells transfected with NOP inhibit adeynlyl cyclase in the presence of an agonist, similar to other opioid receptors. (Meunier et al., 1995; Reinscheid et al., 1995) *In situs* published for the zebrafish embryo are very poor, so the expression of OPRL1/NOP in retinal axons is unclear.(Macho Sanchez-Simon and Rodriguez, 2009) Thus, we are not considering NOP/OPRL as candidates at this time.

Adhesion GPCRs: Latrophilin3 and CELSR3

The single cell expression profiling data identified two members of the adhesion GPCR family – Latrophilin3 and CELSR3/flamingo, as highly expressed in RGCs during development. Adhesion GPCRs comprise a large family (33 members each with

numerous splice isoforms), with a large n-terminal tail decorated with adhesion motifs including EGF-, leucine rich- and cadherin- repeats as well as Ig domains. (Bjarnadóttir et al., 2007; Paavola and Hall, 2012) These receptors are translated as one long protein, which subsequently undergoes proteolytic cleavage at the conserved GPS domain, so the adhesion portion of the receptor is non covalently bound to the GPCR like portion of the receptor (Krasnoperov et al., 2002). The 7TM domains bear some homology with GPCRs and one such receptor, Latrophilin 1, co-purifies with GαO in two-step affinity chromatography – but thus far the role of hetertorimeric g protein signaling in Adhesion-GPCR function is unclear. (Lelianova et al., 1997)

Latrophilin 3 (LPHN3): Adhesion GPCR family member LPHN3 was identified in the Cepko retinal gene expression data set. (Trimarchi et al., 2007) LPHNs 1-3 were initially identified as binding partners for the black widow spider venom toxin α-latrotoxin, which precipitates massive presynaptic neurotransmitter release in affected neurons. (Ichtchenko et al., 1999; Südhof, 2001) LPHN3 is highly expressed in the nervous system, and is thought to signal via GαO, by analogy to LPHN1 (Lilianova et al., 1997). A worldwide linkage-study based analysis of several families related to a genetic isolate in South America, (the Paisa), recently implicated in LPHN3 in ADHD. (Arcos-Burgos et al., 2010) A recent paper used locomotion as a proxy for hyperactivity to study the function of LPHN3 in the developing zebrafish. (Lange et al., 2012) The authors identified two zebrafish paralogs of Latrophilin 3 – LPHN3.1 and LPHN3.2 Both paralogs are broadly expressed in the brain and clearly expressed in the RGC layer at 48hpf, with expression maintained until 4dpf. (Lange et al., 2012). Morpholino knockdown of LPHN3 increased motor activity and also altered the pattern of tyrosine hydroxlase staining in

6dpf embryos, suggesting that dopaminergic neuronal patterning or migration was impacted by the LPHN3 gene.

CELSR3/Flamingo (CELSR3): The second adhesion GPCR identified by the expression screen was CELSR3/Flamingo. CELSRs are so named for their **C**adherin and **E**GF-**L**AG **S**even Transmembrane **R**eceptors. CELSR3 was originally identified as *flamingo* (*fmi*) in a degenerate PCR screen for Drosophila cadherin homologues, and near-simultaneously as *starrynight* (*stan*) in an independent screen for polarity genes (Iwai et al., 1997; Chae et al., 1999; Usui et al., 1999)

CELSR3/fmi/stan features an atypical c-terminus that does not bind cadherin's classical signal transducing partners, catenins. (Usui et al., 1999) Expression of f*lamingo* in S2 cells caused cell aggregation, suggesting that it can promote homophilic cell adhesion. (Usui et al., 1999) Another *flamingo* allele was identified in a screen for defective optomotor response, and mutant photoreceptors R1-R6 project to inappropriate laminae within the optic lobe. (Lee et al., 2003) CELSR3 null mice display severe axon guidance defects, including a lack of anterior commissure, but do not display PCP like phenotypes such as neural tube closure defects. (Tissir et al., 2005; Zhou et al., 2008) At least some of CELSR3's axon guidance effects do not depend on adhesion, as the extracellular domain of *starrynight* is dispensible in mediating the sensory axon pathfinding defects in drosophila. (Steinel and Whitington, 2009) In the zebrafish retina, CELSR3 is expressed in amarcrine cells as well as RGCs, and CELSR3 mutants fail to perform the optokinetic response (OKR). (Lewis et al., 2011)

Considering the expression data, and their established roles in cell migration and polarity, axonal pathfinding, and neuro-behavioral disorders, LPHN3 and CELSR3 are interesting candidates to mediate retinal axonal pathfinding. It's also unclear how this class of 7TM receptors signal – so exploring the role of heterotrimeric G protein signaling

in adhesion GPCRs would be very interesting to the field in general. We have obtained the CELSR3 mutant line, and are in the process of evaluating the mutants for retinal guidance defects. These mutants have a stop codon early in the first exon and are presumed to be nulls. (Lewis et al., 2011) Embryogenesis was reported grossly normal, though swimbladders fail to inflate, I observed that some embryos display heart edema, and a shortened body axis, suggesting these animals may have a covergence/extension planar cell polarity phenotype. (Solnica-Krezel et al., 1996) CELSR3 mutants were identified by their defect in the optokinetic response (OKR). Since OKR is not mediated by the tectum, CELSR3 might direct axons to other retinal arborization fields. (Burrill and Easter, 1994; Roeser and Baier, 2003) Alternatively, CELSR3 might function within RGC dendrites to refine RGC circuitry within the retina, similar to other protocadherins. (Lefebvre et al., 2012). As the role of G protein signaling in adhesion GPCRs is almost wholly unexplored, it would be interesting to see if transiently expressing specific dnGα's potentiate phenotypes of CELSR3 heterozygotes or LPHN3 morphants. If they are observed to have pathfinding defects independent of heterotrimeric G protein signaling, we might explore the role of PCP pathway members using the mutants and reagents already in use in the Granato lab in the dissection of MUSK/*unplugged*'s role in neuromuscular synapse formation. (Gordon et al., 2012)

GPCRs already known to function in axon guidance.

A final class of potential candidates consisted of GPCR signaling pathways already implicated in axon guidance. These included the sonic hedgehog (Shh), CXCR4, and PCAP/PAC1 signaling pathways.

Smoothened (Sonic Hedgehog):

Best known as a morphogen, Shh classically mediates transcription through a canonical pathway, in which Shh binding to co-receptor patched (Ptc) activates the 7TM protein smoothened, resulting in the downstream transcription of Gli target genes. Shh also acts to guide commissural and retinal axons through a non-canonical, smoothened-dependent pathway via Src family kinases. (Charron et al., 2003; Sánchez-Camacho and Bovolenta, 2008; Yam et al., 2009; Fabre et al., 2010; Gordon et al., 2010) Smoothened is a 7TM protein, but a role for heterotrimeric G protein signaling in Shh signal transduction was only recently identified. GTPγS binding assays were carried out in heterologous cells expressing specific heterotrimeric G proteins in the presence or absence of smoothened, and demonstrated that Smoothened activates GαI family members GαI1, GαI2, GαI3, and Gαz but does not activate GαQ, GαS, or G12 family heterotrimeric G proteins. (Riobo et al., 2006) This study is consistent with a recent paper which explored the function of G protein signaling in mediating canonical hedgehog signaling in the drosophila wing. RNAi based studies demonstrated that inhibiting GαI decreased expression of a Ptc-Luciferase reporter, while expressing a constitutively active Gαi, rescues the wing patterning defects of embryos expressing a dnSmo. (Ogden et al., 2008) Taken together, these studies suggest that, while Shh guides retinal axons through a non-canonical pathway – that pathway does not involve GαS.

CXCR4: The chemokine SDF, signaling through the GPCR CXCR4 plays important roles in cell motility including neuronal migration and axon guidance. (Cho and Miller, 2002) (Cho and Miller 2002) SDF can act as an attractant for cerebellar granule cells,

and repels Xenopus spinal axons in turning assays. (Zou et al., 1998; Xiang et al., 2002) CXCR4 is highly expressed in RGCs as they extend axons out of the eye. CXCR4 morphant and mutant RGCs make intra-retinal guidance errors, and fail to project towards the optic stalk, where SDF is expressed, suggesting it may act as an attractant or anti-repellent in this context.(Li et al., 2005) We have extensively characterized CXCR4 in its role as an anti-repellent, and have recently demonstrated that its effects are mediated by GαI, GαQ and Gβγ. (Twery and Raper, 2011) In our previous studies, we also demonstrated that CXCR4 antagonizes Slit/Robo signaling in the optic tract; such that CXCR4 knockdown rescues the guidance defects of the zebrafish Robo2 hypomorph Astray^{te284}. (Chalasani et al., 2007) However, our lab and others have never observed a retinal midline crossing phenotype when CXCR4 is knocked down by morpholino. (Chalasani, Sabol and Raper unpublished observations)

Pac1: The GPCR Pac1 activates GαS upon binding its ligand, the neuromodulator pituitary adenylate cyclase-activating polypeptide (PACAP). Xenopus spinal axons express Pac1, and turn towards a gradient of PACAP through a cAMP and PKA dependent mechanism. (Guirland et al., 2003) The expression patterns of PACAP and Pac in the zebrafish embryo were recently published. While the receptors Pac1b and Pac1b are expressed in spinal cord and habenula, they are excluded from the eye. (Alexandre et al., 2011) Thus, signaling via the Pac1 receptor cannot contribute RGC midline crossing.

In summary: considering a broad range of possible receptors has led me to identify four candidates that we are in the process of testing: the Adenosine2b receptor, mGlur1b, GRMA, and CELSR3. We have excluded some candidates by knockdown, mutant

analysis, or expression studies: the cannabinoid receptor CB1, GABAB1, and CXCR4. (Dell and Raper, Taku and Raper, Chalasani, Sabol and Raper, unpublished observations). We have also been able to exclude some candidates that initially seemed very attractive, such as Smoothened, Pac1, and the D2 dopamine receptor. In the light of this list its also important to consider that the activity of several receptors might be required to maintain GαS mediated cAMP levels in extending retinal ganglion cells.

Part 2: cAMP regulation of Neuropilin Expression

The study presented in Chapter 3 demonstrates that cAMP signaling in RGCs promotes midline crossing by increasing expression of Neuropilin1. There are several open questions related to this study that might be interesting to explore including: **(1)** How does cAMP regulate neuropilin levels? **(2)** What receptor partner(s) cooperate with Neuropilin1, Sema3D and Sema3E to promote crossing? **(3)** How does Sema3D/3E signaling at the midline promote crossing?

How does cAMP regulate Nrp1 expression levels?

PKA? Or EPAC?: PKA is a canonical downstream effector of cAMP. However, EPAC can also be directly activated by cAMP to promote changes in gene expression, mobilize calcium stores, or signal via p38 MAPK. (Ster et al., 2007) To determine if PKA mediates the guidance defects of dnGαS and ADCY8 morpholino, I could perform interaction studies similar to those described in the previous chapter, using a dominant negative PKA in conjunction with subthreshold doses of dnGαS or ADCY8 MO. We have a dnPKA, in which the R1 regulatory subunit of PKA is no longer activated by cAMP. (Clegg et al., 1987) This construct has previously been used in zebrafish and produces

errors in olfactory guidance. (Yoshida et al 2002) We have cloned dnPKA into our Tol2:UAS:MCS:UAS:GAP43:citrine vector and expressed it in retinal axons. In transients, my initial immunohistochemistry based studies revealed minor RGC errors near the chiasm, and confirmed the olfactory guidance phenotype observed by Yoshida and colleagues. However, when we generated stable UAS:dnPKA transgenics, no ipsilateral misprojections were observed in these animals (0/60 projections). Either the UAS was silenced, the dominant negative was not expressed at a high enough level to abrogate PKA signaling, or PKA might not play a role in midline crossing. It would be interesting to revisit these experiments using transient expression of dnPKA in the eye removal assays described in the previous chapter. We could validate the efficacy of the dnPKA by looking for the olfactory guidance defects previously reported, and also examining levels of phospho-CREB in by western blot in WT and dnPKA expressing animals, assuming commercial anti-phospho-CREB antibodies work on zebrafish protein. If the dnPKA is working to reduce CREB levels, we can look for interactions with ADCY8 morphants and dNGαS transgenics using the half-dose morpholino experiments described in the previous chapter. Finally, a constitutively active PKA should rescue the ipsilateral misprojections in dnGαS transgenics or ADCY8 morphants. If the reagent is working, and we don't see an interaction with ADCY8M - then an EPAC mediated pathway might be producing these effects.

Does the effect on Nrp1 expression levels occur via CREB? Similar to the approach described in Imai et al (2006), we should be able to rescue the crossing phenotype by expressing a constitutively active CREB in RGC axons. (Imai et al., 2006) If caCREB rescues the guidance phenotypes observed in Nrp1a or ADCY8 morphants then we would conclude that cAMP's effect on Nrp1 is CREB mediated. If we don't observe a

rescue, a next step would be to try other bZIP transcription factors such as CREM or ATF.

Does cAMP-dependent regulation of Nrp1a occur at the Nrp1a promoter? Our results are consistent with observations in other systems that link cAMP signaling with Neuropilin1 expression. (Jassen et al., 2006; Imai and Sakano, 2007; Yamamizu et al., 2009; Henion et al., 2011) However, the mechanism by which this occurs remains unclear. As mentioned above, an assumption in the field is that cAMP promotes CREB dependent transcription at the Nrp1a promoter. One way to test this assumption would be to carry out ChIP assays in WT and cAMP-inhibited or activated neurons over a limited time course. It has proved technically challenging to culture zebrafish axons. For these experiments, we could turn to a neuronal cell line. Consistent with our results, Forskolin mediated cAMP increase promotes neuropilin expression in SK-N-MC human neuroepithelioma cell line. (Jassen et al 2006) If we were do extensive ChIP it might make sense to do it in this cell line. We might also carry out these experiments in non-zebrafish primary neuronal culture. Though chick RGCs do not express Neuropilin1, Sema3A sensitive DRGs do express the receptor. If ChIP assays do not reveal CREB at the Nrp1 promoter, another transcription factor could be mediating the effect at the Nrp1 promoter, or CREB might be acting at some other promoter to indirectly upregulate Nrp1 expression.

How does Sema3D and 3E signaling promote midline crossing?

Which Plexins co-operate with Nrp1a and Sema3D and Sema3E to facilitate midline crossing?

Neuropilin is only one component of the class 3 Semaphorin holoreceptor complex, which includes Plexins and may include IgCam adhesion molecules such as L1 and

NrCAM. (reveiwed in Pasterkamp, 2012) One outstanding question for the results presented in chapter 3 is, which Plexin(s) transduce Sema3D and 3E signals at the midline. We thus examined the expression patterns of zebrafish PlexinA1a, PlexnA1b, PlexinA2, PlexinA3, PlexinA4, PlexinB1a, Plexinb1b, PlexinB2, and PlexinD1. (Xu and Raper, unpublished observations) We detected expression of all of these Plexins, except PlexinA4 and PlexinB1a, in the RGC layer at 36hpf, consistent with previous reports. (Christie et al., 2006). Thus, expression analysis of the vertebrate plexins did not reveal an obvious candidate for a role in Sema3D/3E mediated RGC guidance. We also analyzed the retino-tectal projections of *sidetracked* (PlexinA3) mutants. (Palaisa and Granato, 2007) We did not observe ipsilateral misprojections in these animals (0/26 projections analyzed). (Fried-Cassorla and Raper, unpublished observations) Tectal borders seemed to be shifted towards the midline in mutants, suggesting that PlexinA3 may mediate later stages of RGC guidance. We also performed knockdown experiments targeting PlexinA1 with two different morpholinos. We observed very few minor ipsilateral projections (8/110) with translation blocking and with high doses of splice blocking MO (3/40), but these do not account for the scope of errors observed in Sema3D, Sema3E, Nrp1a, or ADCY8/dnGαS loss of function experiments. (Xu and Raper, unpublished results) In mouse, Sema3E acts as a repellent for corticospinal axons when signaling through PlexinD1, but an attractant for subiculo-mammilary neurons when signaling through PlexinD1 and Nrp1.(Chauvet et al., 2007) PlexinD1 is expressed in RGCs at 36hpf, but analysis of PlexinD1 mutants did not reveal any ipsilateral misprojections (0/22). (Torres-Vázquez et al., 2004) We surmise that multiple Plexins may contribute to Sema3D/Sema3E mediated axon guidance in navigating RGCs, consistent with previous reports that Plexins can act redundantly. (Schwarz et al., 2008)

IgCAMs L1 and NrCAM are also components of class 3 semaphorin receptor signaling, which might contribute to Sema3D and Sema3E mediated RGC crossing. In explant culture, L1 -/- cortical axons are insensitive to sema3A. (Castellani et al., 2000) Sema3D promotes expression of L1 to contribute to fasciculation in the zebrafish MLF (Wolman et al., 2007) NrCAM can act on its own to promote outgrowth, and in stripe assays chick RGCs preferentially grow on NR-CAM surfaces, consistent with a role as an attractive or permissive cue, though in commissural axons this effect seems also to require NgCAM and Axonin-1. (Fitzli et al., 2000; Zelina et al., 2005) The SAGE expression data from the Cepko lab confirmed that L1 and NrCAM are expressed on developing mouse RGCs, and all are expressed in the zebrafish retina at 3dpf. (Tongiorgi et al., 1995; Bernhardt et al., 1996; Trimarchi et al., 2007) In the mouse, RGC neurons that are fated to project ipsilateraly are born in the ventral temporal retina, while ventral temporal RGCs project contralaterally. (Herrera et al., 2003) DT explants extend longer neurites than their ipsilateral VT counterparts when cultured near chiasm explants, and this effect is abolished by treatment with anti-Sema6D antibodies, suggesting Sema6D acts as an attractive or permissive cue to promote midline crossing. Surpisingly, DT axons are repelled by Sema6D when cultured near HEK cells expressing Sema6D, suggesting that other factors in the chiasm contribute to the permissive effect. The permissive switch is mediated by interactions between NrCAM on RGCs and on midline glia and PlexinA1 on neurons cells near the chiasm. (Kuwajima et al., 2012) NrCAM is also required for Sema3F mediated repulsion of cortical axons (Falk et al., 2005)(Falk et al 2005) In our system NrCAM is expressed on RGCs, but its expression pattern at the zebrafish midline is unknown. Thus NrCAM could facilitate Sema mediated midline crossing by promoting a permissive interaction with Sema3D and Sema3E at the midline, or could

act to mediate repellent signals that promote post-crossing axons leaving the midline. The latter model would require tight temporal control of Nrp1 expression at the growth cone, raising the question of weather cAMP contributes to temporal regulation of Nrp1.

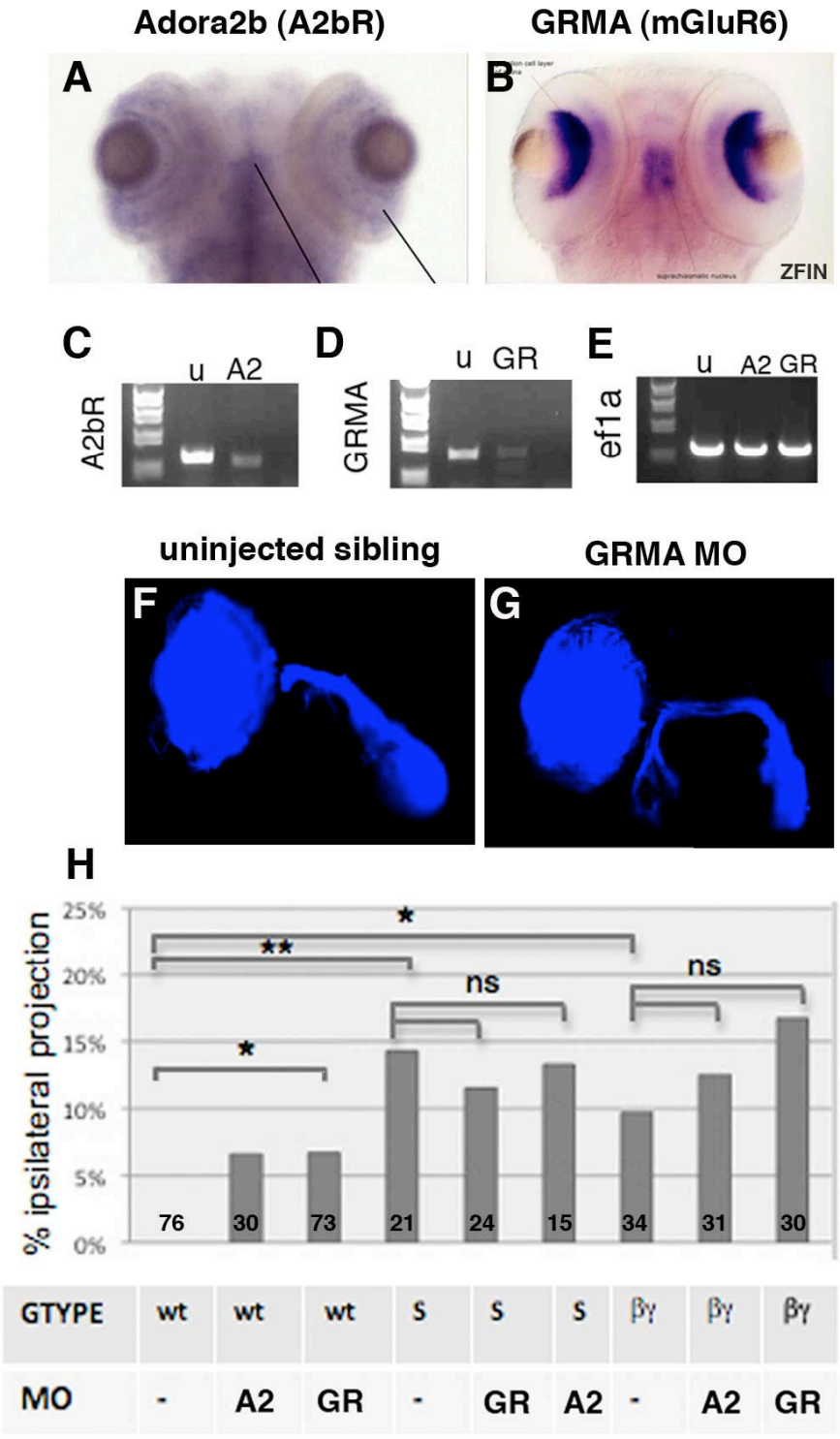
How are cAMP and Nrp1 levels regulated temporally?

In Xenopus, retinal axons require Neuropilin for later pathfinding events in the optic tectum. In this system, precrossing axons do not collapse in response to Sema3A and Nrp1 isn't expressed until axons are crossing the midline, consistent with A5 antigen (Nrp1) immuoreacitivity. (Takagi et al., 1991; Campbell et al., 2001) This presents the intriguing possibility that upregulation of Nrp1 might occur as navigating axons respond to environmental cues or via spontaneous activity to promote calcium or cAMP levels. (reviewed in Borodinsky and Spitzer, 2006) In Xenopus RGCs, temporally regulated expression of Nrp1 occurs even in cultured axons. (Campbell et al., 2001) The authors interpreted this result as an argument against the presence of an external signal mediating Nrp expression, and have since implicated miRNA124 in temporal control of Nrp1 expression. (Baudet et al., 2012) In the context of our work, GPCR signaling may contribute to cAMP levels through basal activity of its receptors, similar to olfactory system. (H. Sakano personal communication) Alternatively, since these experiments were carried out in explant cultures, extrinsic GPCR mediated signaling could occur through interactions with sibling RGCs grown in culture. Our own experiments do not address cAMPs role in temporal control of Nrp1 expression as our interventions with cAMP signaling occur early; the dnGαS transgene is expressed under control of Ath7, which is required for the specification of RGCs, and ADCY8 morpholino knockdown is carried out at the one cell stage. (Kay et al., 2001) I re-examined the single cell expression data from the Cepko lab's SAGE analysis, and (within considerable variability between cells of the same age) did not observe a temporally correlated change in Nrp1

expression levels between e12 and e15 (the time that RGC axons approach and cross the midline), though it does seem to be downregulated by p0. (Williams et al., 2004; Trimarchi et al., 2007) Thus cAMP might regulate Nrp1 expression early or late. It might be interesting to perform a time course analysis to study expression levels of Npr1 in RGCs together with surface labeling experiments to determine when Nrp1 is expressed on the growth cone. One nice experiment would be to use the pH sensitive GFP(pHLuorin) together with live imaging to track Nrp1 expression on the surface of RGC growth cones in real time. (Nawabi et al., 2010)

GPCR signaling stands at a fascinating intersection of cell adhesion, control of cyclic nucleotides and calcium dynamics, and gene expression. We have identified a role for GαS coupled GPCR signaling in promoting expression of the axon guidance receptor Nrp1, thus interacting with a classic axon guidance pathway. However, ultimately I wanted the dnGα reagents to lead to the identification of novel receptors and ligands that contribute to the formation of neuronal circuits in the developing embryo. One concern with these reagents is that they might not be expressed at high enough levels to fully abrogate Gα signaling. However, this might turn out to be an advantage in identifying G protein partners for uncharacterized GPCRs either associated with a specific mutant phenotype, or expressed in a neuronal population or axonal projection of interest. dnGα's can be used to attenuate G protein signaling in heterozygous backgrounds and look for enhancement of any observed phenotypes. With the increasing availability of Gal4 lines, and insertional mutants, UASdnGα and βγ constructs can be used to probe G-protein function in the context of specific neuronal populations and candidate GPCRs.

Figure 4.1


Adora2b (A2bR)
GRMA (mGluR6)
A
B
ZFIN
C
u A2
A2bR
D
u GR
GRMA
E
u A2 GR
ef1a
uninjected sibling
GRMA MO
F
G
H
% ipsilateral projection
25%
20%
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10%
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76
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GTYPE wt wt wt S S S βγ βγ βγ
MO - A2 GR - GR A2 - A2 GR

Figure 4.1. Adora2b and GRMA, candidate GPCRs which direct retinal axon crossing

The Adenosine2b receptor Adora2b is expressed in the zebrafish retina (Boehmier et al. 2009), B. GRMA is expressed in the zebrafish retina. (ZFIN) C-E. Morpholino knockdown destabilizes the Adora2b (C) and GRMA (D) transcripts, EF1a serves as a control to demonstrate equal template loading between samples (E) F. Retinal axons of uninjected siblings project contralaterally. G. RGCs of GRMA morphants project ipsilaterally, similar to GαS and Adora2b knockdown (not shown). H. Knockdown of GRMA or Adora2b in transgenics expressing either dnGαS or dn$\beta\gamma$ *=$p<0.05$, **=$p<0.01$ by fisher exact test.

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