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Axon target matching in the developing visual system

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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2015
The Dissertation of Jessica A. Osterhout is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2015
DEDICATION

For my family who has supported me at every step of my academic career. Thank you for always telling me how proud you are and encouraging me to pursue my PhD.

And for my husband Jared Sewell, my scientific colleague and unwavering supporter. You inspire me to be better at everything I do.
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<td>AOS</td>
<td>accessory optic system</td>
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<td>Cdh3</td>
<td>cadherin 3</td>
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<td>Cdh6</td>
<td>cadherin 6</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNTN4</td>
<td>contactin 4</td>
</tr>
<tr>
<td>CTβ</td>
<td>Cholera toxin subunit β</td>
</tr>
<tr>
<td>CTX</td>
<td>cortex</td>
</tr>
<tr>
<td>dLGN</td>
<td>dorsal lateral geniculate nucleus</td>
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<tr>
<td>DRD4</td>
<td>dopamine receptor D4</td>
</tr>
<tr>
<td>GCL</td>
<td>ganglion cell layer</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HoxD10</td>
<td>homeobox D10</td>
</tr>
<tr>
<td>IGL</td>
<td>intergeniculate leaflet</td>
</tr>
<tr>
<td>INL</td>
<td>inner nuclear layer</td>
</tr>
<tr>
<td>IPL</td>
<td>inner plexiform layer</td>
</tr>
<tr>
<td>ISGS</td>
<td>lower stratum griseum superficialis</td>
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<tr>
<td>mdPPN</td>
<td>medial division of the posterior pretectal nucleus</td>
</tr>
<tr>
<td>MTNd</td>
<td>dorsal medial terminal nucleus</td>
</tr>
<tr>
<td>MTNv</td>
<td>ventral medial terminal nucleus</td>
</tr>
<tr>
<td>NOT</td>
<td>nucleus of the optic tract</td>
</tr>
<tr>
<td>OC</td>
<td>optic chiasm</td>
</tr>
<tr>
<td>ON</td>
<td>optic nerve</td>
</tr>
<tr>
<td>OPN</td>
<td>olivary pretectal nucleus</td>
</tr>
<tr>
<td>PR</td>
<td>photoreceptor</td>
</tr>
<tr>
<td>RGC</td>
<td>retinal ganglion cell</td>
</tr>
<tr>
<td>SC</td>
<td>superior colliculus</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
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<tr>
<td>SO</td>
<td>stratum opticum</td>
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<tr>
<td>TRHR</td>
<td>thyrotropin-releasing hormone receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>--------------------------------------</td>
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<tr>
<td>uSGS</td>
<td>upper stratum griseum superficialis</td>
</tr>
<tr>
<td>vLGN</td>
<td>ventral lateral geniculate nucleus</td>
</tr>
<tr>
<td>ZI</td>
<td>zona inserta</td>
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PUBLICATIONS


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Major Field: Developmental Neurobiology
ABSTRACT OF THE DISSERTATION

Axon target matching in the developing visual system

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University of California, San Diego, 2015

Professor Andrew Huberman, Chair

The central nervous system (CNS) is made up of trillions of connections between specific sets of highly specialized neurons. How each individual neuron finds and connects to the correct synaptic partner remains an important and unresolved issue in neuroscience. Using the mouse visual system as a model I probed the cellular and molecular mechanisms that govern one of the key steps leading to CNS development: axon target matching. Axon target matching is the process by which axons to find and innervate their correct target nuclei in the brain. I focused on eye-to-brain connections made by retinal ganglion cells (RGCs). First, I discovered that RGC birthdate correlates with the timing of axon out growth from the
eye to the brain and that the timing of axon arrival predicts the mode of axon target matching. The earlier an RGC axon innervates the brain, the more targets it innervates and ultimately the more axonal refinement must take place before it reaches the final wiring pattern. Conversely, the later an RGC axon innervates the brain the more likely it will project to only the correct targets and undergo minimal refinement. Second, I discovered that specific adhesion molecules expressed by RGC axons and/or the cells in their target nuclei are required for correct axon target matching. These include cadherin-6, contactin-4 and amyloid precursor protein. I found that a loss of a single adhesion molecule and thereby the loss of connections made by a single functionally-specialized category of RGCs to the brain, results in system-wide defects in specific visually-driven behaviors. My results shed light on how sensory neurons in the mammalian visual system rely on timing of cell birth and axon outgrowth, along with specific cell adhesion molecules to form highly specialized long-range connections required for correct visually-driven perception and behavior. These results speak to possible general mechanisms of neural development in the CNS.
INTRODUCTION

Neural circuits are the basis for environmental perception, information processing, and behavior. In the central nervous system (CNS) different types of functionally and anatomically specialized neurons communicate information to each other through neural circuits spanning microns to millimeters. This transmission of information is highly specific and the specificity is crucial for the function of the CNS. In the late 18th century the German physician, Franz Joseph Gall proposed that each localized region of the brain has a precise function. We now know that specific regions of the brain, or nuclei, are indeed functional specialized and that they receive specific information that they relay to other stereotyped brain regions, thereby forming neural circuits. Processing of neural information appears more complicated than the work of Gall predicted, however, as many different nuclei are intricately interconnected and many contain specialized layers, subdivisions and heterogeneous populations of cell types. At the same time, it is clear that precise connections between neurons from different specialized locations are crucial for proper nervous system function.

Forming long-range connections

In order to form long-range connections, neurons whose cell bodies remain relatively stationary must send their axons out to eventually find and form synapses with the correct post synaptic partner cell. To do so, each axon must i) select and follow the correct trajectory or axon tract, ii) depart the tract to innervate the correct target nuclei, iii) find the correct layer within the target nucleus and finally iv) connect to the correct cell type. The program of cellular and molecular mechanisms required to complete these processes is still
being uncovered.

A large body of work has demonstrated that axons respond to molecular guidance cues in either an attractive or repellent manner (reviewed in Dickson, 2002). These molecular cues initially guide axons along the correct trajectory en route to their targets. One of the first and most famous examples of long-range guidance by a diffusible signal is the discovery of Netrin-1, a molecule expressed in the ventral spinal cord. Marc Tessier-Lavigne and co-workers showed that Netrin-1 is expressed by cells in the floor plate of the chick spinal cord and attracts dorsal commissural axons towards the floor plate during embryonic development (Kennedy et al., 1994). Netrin-1 was identified through a large-scale biochemical purification and an in vitro screen of purified molecules which were added to cultured spinal neurons to test their impact on neurite outgrowth. Many axon guidance molecules were initially discovered in such in vitro experiments. Now several decades later, we have a good grasp on the identity and action of ligands and receptors that govern general chemoattraction and repulsion of long-range axon guidance (reviewed in Tessier-Lavigne and Goodman, 1996 and Dickson, 2002). It is worth noting however that because developing vertebrate brains are large, three-dimensional structures containing various neurons, and extracellular matrices for axons to navigate through, broad diffusible molecules alone are not sufficient to establish complex neural pathways and intricate wiring patterns. Thus, a complex program of cellular and molecular mechanisms has evolved to ensure specificity of connections during CNS development.

**Directed growth and axon tracts**

Axon guidance molecules expressed by specific subsets of neurons combine with
extracellular matrix factors to create axon tracts through which many different axons can travel. For example, the membrane-bound cell adhesion molecules L1 and N-cadherin and the extra-cellular matrix component laminin serve as substrates to guide axons down general axon tracts but notably, they do not provide directional information (Lemmon et al., 1992). In addition, repellant molecules such as the slits and its receptors, the robos, can dictate the boundaries of axon tracts by repelling robo-expressing axons away from slit-expressing boundaries (Ricano-Cornejo et al., 2011). Tract formation also requires specific cellular and molecular programs; a recent study provided evidence that thalamo-cortical tract formation requires accurate tangential migration of a population of neurons from the ventral telecephalon (Lopez-Bendito et al., 2006). That study showed that neurons migrate to the correct location and begin expressing two particular molecules, CRD-NRG1 and Ig-NRG1, that together guide axons down the thalamo-cortical tract. Thus, general molecular programs exist to generate axon tracts, which guide long-range axons to different regions of the brain.

As an axon growth cone travels down a tract towards its target, it often encounters multiple ‘choice-points’ at which it must project in one direction or the other. Such choices are often mediated by guidepost cells, which locally express molecular cues. In C. elegans, small invertebrate worm with a relatively simple nervous system, synaptic guidepost cells in the vulval epithelium express the cell adhesion molecule SGY-2, which directs synapse formation onto their correct axonal target. Loss of SGY-2 reduces synapses on the normal target and causes ectopic synapses onto inappropriate axons (Shen et al., 2004). In vertebrates, repellent interactions mediated by Ephrin-B and its receptor EphB as well as attractive interactions mediated by L1 and CD44 instruct retinal ganglion cells (RGCs), the output neurons of the eye, to project to one side of the brain or the other at a choice point
called the optic chiasm. As first demonstrated in *xenopus*, Eph-B expressing RGCs are repelled away from the midline by Ephrin-B-expressing cells at the chiasm and project ipsilaterally to targets in the brain (Nakagawa et al., 2000). Several years later Mason and co-workers discovered that in mammals, Ephrin-B2 and EphB1 interact similarly to repel ipsilaterally-projecting RGC axons from the midline at the chiasm in mice (Williams et al., 2003). Conversely, L1-expressing RGCs that encountering CD44-expressing cells at the optic chiasm cross the midline and project contralaterally to their target nuclei (Sretavan et al., 1994).

Some choice points also serve as intermediate targets, in which axons wait for a particular developmental stage before proceeding and require additional signals to move on from that target. For example, in zebrafish, *chodl* is expressed by the horizontal myoseptum, an intermediate target and choice point for motor neurons; when *chodl* is knocked down by morpholino application, motor axon growth is arrested or stalled at the myoseptum leading to reduced muscle innervation (Zhong et al., 2012). Like the formation of axon tracts, the formation of choice points is highly regulated; in the zebrafish mutant unplugged, some motor neurons make incorrect decisions at their set choice points but interestingly, *unplugged* is expressed in cells adjacent to the choice point and before axon arrival and thus has no contact with the motor neurons (Zhang et al., 2004). The group discovered that unplugged signaling is required to induce changes in the extracellular environment necessary for mediating correct axonal pathfinding at those choice points (Zhang et al., 2004). Together, studies such as these suggest that axon guidance is often controlled by local cues that are expressed by guidepost cells to direct axons down different trajectories and towards their targets.
**Growth guided by axon-axon signaling**

In order to find and project to the correct regions of the brain, axons also utilize signals from neighboring axons. Axon-axon mechanisms for axon guidance include pioneer/follower axons, and selective fasciculation with axons of similar or different origin. First discovered in grasshoppers, pioneer axons grow from the epidermis towards the CNS early in development and are later followed by sensory neurons (Bate, 1976). If the pioneer axons are lost then these sensory neurons fail to reach the CNS (Klose and Bentley 1989). In the mammalian brain, early pioneer axons traverse from the cortex to the thalamus before the cortical neurons that will eventually project toward subcortical structures are generated (McConnell et al., 1989). Pioneer neurons typically originate from the same structure as the follower axons but axons can also be guided by afferents originating from the target structure itself. For example, a recent study showed that dopaminergic axons projecting to the lateral habenula were shown to require contact with outgrowing habenular axons and if habenular neurons are lost, the dopaminergic axons fail to grow toward this target (Schmidt et al., 2014). Similarly, ingrowth of thalamo-cortical axons is required to guide pioneer cortico-thalamic axons to the correct trajectory (Deck et al., 2013).

Developing axons must be able to interact with either pioneer axons or target-derived axons in order to follow them to the correct target. Axons can selectively fasciculate with other axons with cell adhesion molecules expressed along their length. Regulation of these adhesion molecules is required to selectively fasciculate or de-fasciculate with the right axon partners. In the mouse olfactory system, adhesion molecules such as Kirrel2/Kirrel3 and contactin-4 (CTN4) as well as repellent molecules such as Ephrin-A5/EphA5 are required for
olfactory sensory neurons (OSNs) carrying the same olfactory receptor to fasciculate only with like OSNs and ultimately to reach the correct target glomeruli in the olfactory bulb (Serizawa et al., 2006; Kaneko-Goto et al., 2008). Similarly, motor neuron axons projecting to either fast or slow twitch muscles selectively fasciculate together before innervating the correct muscle type (Milner et al., 1988). It is equally important that an axon can de-fasciculate from the axon bundles at the correct locations. The proteins FasII and Beat are required for selective de-fasciculation in insects; loss of either of these results in mis-targeting phenotypes in motor neurons (Lin and Goodman, 1994; Famborough and Goodman, 1996). Thus, selective fasciculation with pioneer axons, axons of similar type or target-originating axons likely reduces the need for extra guidance molecules and increases the efficiency and accuracy of circuit formation.

**Pre-target sorting**

Another mechanism to increase the targeting efficiency and accuracy of axons within a circuit is pre-target sorting of axon bundles before they reach their target structure. This may be especially relevant in circuits containing many different axons projecting to a multi-function target. Indeed, the axons of olfactory sensory neurons (OSNs) are pre-sorted into region specific populations before entering the olfactory bulb (Miller et al., 2010). In mice, OSN axons express varying levels of neuropilin-1, which interacts in a repellent manner to target-derived semaphorin-3A; OSN axons are sorted in a gradient according to how much neuropilin-1 they express (Imai et al., 2009). A similar phenomenon occurs in the avian auditory system whereby axon trajectories are organized according to topographic location, suggesting that pre-target sorting contributes to precise topographic location in the auditory
brainstem (Kashima et al., 2013). In addition, RGCs axons are pre-sorted, according to the location of the cell body in the retina along the dorsoventral axis, before they innervate the superior colliculus and therefore long before the maturation of the topographic map in the target itself (Plas et al., 2005).

**Target-directed branching**

Once in the vicinity of a target, an axon growth cone can project into that target and elaborate terminal axon arbors. Alternatively, the axon can innervate a target by forming a collateral branch that projects to that target while the parent axon travels further on to a different target. Corticospinal axons form a collateral branch on their way to the spinal cord in response to a still-yet-identified diffusible molecule within the pons (Heffner et al., 1990). In vitro experiments using trigeminal ganglion cells show that axons can either form tracts or arborize depending on contact with different target tissues or age of the tissues, indicating that axon branching is controlled by expression of extrinsic factors in specific tissues (Erzurumlu er al., 1993). *In vivo*, a population of Pax6-expressing cells near the principal mammillary axon tract are essential to induce location-specific axon collateral branching that leads to innervation of the thalamus. Loss of Pax6 results in a loss of the mammillothalamic tract (Szabo et al., 2011). Non-specific branching is likely controlled by mechanisms intrinsic to the projection neuron. The loss of Dscam in *Drosophila* results in multiple branches projecting to any available target and also increased axon branching within those targets (Wang et al., 2002). In addition, Dscam-mediated branching can be modulated in a spatially specific manner by Slit; upon entering the ventral nerve cord, mechanosensory neurons ‘sprout’ many extensions, which are stabilized and form collateral branches upon Dscam-Slit
binding at the midline (Dascenco et al., 2015). Thus, location-specific cues are required for collateral branch formation that ultimately leads to innervation of multiple targets.

**Axon-target matching and choices within targets**

Axon target matching, or the ability to detect which targets to innervate and which to avoid, is a crucial step for circuit formation as each target sends, receives and processes very specific information, which is ultimately required for correct perception, processing and behavior. How is axon target matching accomplished? After traveling down the prescribed axon tracts, an axon encounters many different target nuclei. Until recently much of the work identifying mechanisms for target innervation has been carried out in models like the *Drosophila* where sensory projections are directed to only one or two targets and functional organization arises from sub-target structure. For example, in the *Drosophila* visual system photoreceptors project to specific layers within one of their two main targets, the lamina and the medulla. Many different molecules, such as N-cadherin, LAR and flamingo, have been identified as guidance molecules for drosophila photoreceptors, which are expressed within the target layer of the lamina or medulla (Lee et al., 2001; Clandinin et al., 2001; Senti et al., 2001; reviewed in Mast et al., 2006). Similarly, in the *Drosophila* olfactory system, OSNs form specific connections with a single glomerulus within the antennal lobe with the help of several cues, including toll receptor 6 and 7 (Ward et al., 2015, reviewed in Hong and Luo, 2014). Until recently, very little was known about how axons pick targets in the mammalian brain.

Like other steps in CNS development, there are likely repulsive cues, which prevent innervation of specific targets. For example, in the chick olfactory system, semaphoring-3A
signals prevent olfactory sensory neurons from projecting to the olfactory bulb to early or from exiting the olfactory system (Renzi et al., 2000). Similarly, in drosophila, muscles expressing wnt4 prevent innervation from incorrect motor axons (Treloar et al., 2009). In addition, attractive and repellent cues expressed by cells or neurons within target structures are likely also required in mammalian brains but with the increased number of neural cell types and increased number and complexity of target structures with which to interact, the mechanisms are also apt to increase in complexity.

Selecting a layer

Many targets in the mammalian brain are further subdivided into function-specific layers and after innervating their correct target nuclei, axons must identify and project to their specific laminar target (reviewed in Sanes and Yamagata, 1999). In the zebrafish optic tectum – a structure analogous to the superior colliculus in mammals – several molecules have been shown to be are required for setting up laminar-specific axon projection domains and for directing RGC axons to their functionally-specific layer (reviewed in Baier, 2013). One proposed mechanism for creating laminar organization in the zebrafish optic tectum is a superficial-to-deep gradient of Slit1, which guides Robo2-expressing RGC axons to their correct target layer (Xiao et al., 2011). In the chick optic tectum, specific cadherins are expressed within individual laminae and are required for correct matching between RGC axons and their laminar target (Inoue and Sanes, 1997, Miskevich et al., 1998). Thus, general axon guidance molecules are expressed within target nuclei and are used by axons to zero-in on the laminar location of their synaptic partners.
Cell type choice

Once an axon has innervated the correct target and the correct layer within that target, it must then find the correct cell-type with which to form a synapse. At this stage an axon must be able to distinguish between one cell type over another using a similar molecular toolkit as for all the proceeding steps described above, but on a smaller, more local special scale. How they are able to do accomplish such specificity is still under intense investigation.

One recent example of axon-cell type matching comes from work in the spinal cord and involves matching between complexes of adhesion molecules expressed by different cell types. Recognition and synapse formation between proprioceptive sensory neurons in the periphery and GABAergic inhibitory interneurons occurs in the spinal cord and requires an adhesive protein complex consisting of contactin-5 (also called NB-2) and Caspr4 expressed by the sensory neurons and expression of NrCam and CHL1 by the interneurons (Ashrafi et al., 2014). Thus, future studies of cell-type-specific targeting will likely lead to new molecular mechanisms, which allow for a higher level of specificity.

Basis for studying the process of axon target matching

In the field of neural development, it is clear that the molecular mechanisms of attraction/adhesion and repulsion are a common theme at each spatial scale from trajectory choice to cell-type choice but it is still unclear how the same set of molecules can control each step involved in circuit formation. It’s also apparent that, compared to animals that have smaller nervous systems and fewer cell types, such as *c. elegans* and *drosophila*, there will likely be additional mechanisms required to ensure precise connections are made in mammalian brain. The questions posed and addressed here in the context of the mammalian
CNS are as follows:

1. What are the cellular mechanisms of axon target matching?
2. What are the molecular mechanisms of axon target matching?
3. What are the consequences of disrupting molecular mechanisms of axon target matching?

Below I address these questions in the context of the mouse visual system where over thirty different types of output neurons, the retinal ganglion cells (RGCs), project to the brain and innervate 1-3 of over two dozen possible retinorecipient targets, each serving unique role in perception and behavior. The importance of the matching process for normal vision is tremendous because the mature projection pattern of individual RGC subtypes differs according the function of the circuit they connect to. For example, RGCs carrying information about brightness in the visual field project to targets that are part of the circadian systems or pupil reflex and RGCs that detect motion or contrast connect to targets that are required for image formation. This arrangement provides a unique opportunity to uncover the mechanisms of long-range axon target matching in mammals, and the potential to link how axon target matching governs visually-driven behaviors. Ultimately, my goal is to discern the larger implications about circuit formation in the mammalian CNS for purposes of revealing general principles and possible origins of diseases when these processes go awry.
References


CHAPTER 1: Birthdate and outgrowth timing predict cellular mechanisms of axon-target matching in the developing visual pathway

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Birthdate and Outgrowth Timing Predict Cellular Mechanisms of Axon Target Matching in the Developing Visual Pathway

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SUMMARY

How axons select their appropriate targets in the brain remains poorly understood. Here, we explore the cellular mechanisms of axon target matching in the developing visual system by comparing four transgenic mouse lines, each with a different population of genetically labeled retinal ganglion cells (RGCs) that connect to unique combinations of brain targets. We find that the time when an RGC axon arrives in the brain is correlated with its target selection strategy. Early-born, early-arriving RGC axons initially innervate multiple targets. Subsequently, most of those connections are removed. By contrast, later-born, later-arriving RGC axons are highly accurate in their initial target choices. These data reveal the diversity of cellular mechanisms that mammalian CNS axons use to pick their targets and highlight the key role of birthdate and outgrowth timing in influencing this precision. Timing-based mechanisms may underlie the assembly of the other sensory pathways and complex neural circuitry in the brain.

INTRODUCTION

Neurons carrying distinct categories of sensory information establish highly specific patterns of connections in the brain and thereby link information about the outside world to the appropriate perceptions and actions. The establishment of this connectivity involves many developmental processes, some of which have been intensely studied, such as synapse formation, axon guidance, topographic mapping, and laminar targeting (Dickson, 2002; McLaughlin and O’Leary, 2005; Luo and Flanagan, 2007; McAllister, 2007; Huberman et al., 2010). However, several crucial aspects of neural circuit assembly remain unresolved. An important example is axon target matching: the process by which an axon distinguishes among and innervates specific target structures (Figure S1A). Axon target matching has been explored in detail for invertebrates and within vertebrate spinal circuits (Goodman and Shatz, 1993; Clandinin and Zipursky, 2002; Dasen and Jessell, 2009), but the basic cellular mechanisms that ensure emergence of this feature in the mammalian brain remain poorly understood. Achieving a thorough understanding of the cellular mechanisms for axon target matching is a crucial first step toward establishing molecular models of this process.

Eye-to-brain connections are a potentially powerful model system for probing the mechanisms of axon target matching. They are comprised of retinal ganglion cell (RGC) axons that as a general group all have the same function: to convey visual information to the brain. However, RGCs are highly diverse; they include ~20 subtypes, each responding best to a specific feature in the visual world such as luminance, directional motion, or contrast and projecting that information to a stereotyped collection of target structures in the brain (Dhande and Huberman, 2014). Eye-to-brain connections thus raise the opportunity to explore the development of axon target matching in the context of a brain circuit whose function is known and that includes a variety of cell types, each of which connect to multiple long-range targets residing along the same growth trajectory.

Here, we asked how functionally distinct categories of RGCs find and connect to their targets during development using four transgenic mouse lines, each with GFP selectively expressed in one or two RGC subtypes distributed throughout the retina (Figures S1 and S2; Huberman et al., 2009; Osterhout et al., 2011; Rivlin-Etzion et al., 2011, Dhande et al., 2013). We find that different RGCs employ very different cellular strategies to achieve axon target wiring specificity. Moreover, we find that the mode by which an RGC achieves that specificity systematically varies according to its birthdate and timing of axon ingrowth. These results shed light on the cellular mechanisms used to establish parallel visual pathways and, in doing so, offer a general proposal for how timing of cell birth and axon growth could impact the assembly of complex neural circuits in the brain.

RESULTS

Cdh3-RGC Axons Innervate Their Correct Targets during the Late Embryonic Period

At maturity, Cdh3-RGC axons project mainly to non-image-forming visual targets (Osterhout et al., 2011; Figure S2). When
Figure 1. Cdh3-RGC Axons Innervate the Brain and Undergo Axon Target Matching during Late Embryogenesis

(A) Cdh3-RGCs migrating (yellow arrowhead) to the ganglion cell layer (GCL). Axons are entering the optic nerve head (onh) on E14. L, lens; ON, optic nerve; NFL, nerve fiber layer; d, dorsal; v, ventral. Scale bar represents 500 μm.
and how do Cdh3-RGCs find these targets? Cdh3-RGCs axons were visible in the eye and optic chiasm and optic tract by embryonic day 12 (E12; Figures 1A–1C). On E15, Cdh3-RGC axons were observed in proximity to one of their future targets, the olivary pretectal nucleus (OPN; Figure 1D), but they were not observed within the OPN until E16 (Figure 1E). The density of Cdh3-RGC axons in the OPN increased from E16 to postnatal day 8 (P8; Figures 1E–1G), reaching maximum during the first postnatal week (Figure 1Q, magenta). From P8 to P20, Cdh3-RGC axons underwent slight refinement, such that their overall density of terminations was eventually reduced while the percent of overall target innervation remained unchanged (Figures 1G, 1H, and 1Q).

Cdh3-RGC axons were first detectable in their other major target, the posterior pretectal nucleus (PPN), by E17 (Figure 1I), where from P0 to P8, their terminals aggregated into two dense foci—a hallmark feature of retinal projections to this target (Figures 1J–1L; Osterhout et al., 2011). Together, these data reveal that Cdh3-RGCs begin to innervate their two main pretectal targets during late embryogenesis.

Target Selection by Cdh3-RGCs in the Developing Visual Thalamus
At maturity, Cdh3-RGCs project heavily to the thalamic intergeniculate leaflet (IGL) and ventral lateral geniculate nucleus (vLGN; Figures S2B and S2B’; Osterhout et al., 2011). Cdh3-RGCs innervate these nuclei by E16 (Figures 1M–1P). From E16 through the first postnatal week, Cdh3-RGCs also provided input to the dorsal lateral geniculate nucleus (dLGN; Figures 1M–1O), a target they project minimally to after P20 (Figures 1P, 1Q, and S2B; Osterhout et al., 2011). Interestingly, the route by which Cdh3-RGC axons reached the dLGN underwent dynamic shifts across development. At E16, Cdh3-RGC axons reached the dLGN by projecting dorsally, through the vLGN (Figures 1M and 1N’, arrow). By contrast, at P0, Cdh3-RGC axons sampled the dLGN via trajectories perpendicular to the optic tract (Figures 1N and 1N’, arrow), a configuration that closely resembles the target-entry routes of mature retino-dLGN axons (Dhande et al., 2011). By P8, Cdh3-RGCs also targeted the dorso-medial dLGN (Figures 1O and 1O’). Removal of Cdh3-RGC axons from the dLGN occurred gradually, occupying ~45% of the total target area on P8 and ~5% on P20 (Figure 1Q). Cdh3-RGC projections to the vLGN also diminished during this period (Figure 1Q). Thus, Cdh3-RGC axons select among several neighboring visual target structures in the thalamus by first sampling all of them and then by selectively removing inputs from just one of those targets, the dLGN.

Cdh3-RGCs Axons Initially Overshoot Their Future Targets
Notably, Cdh3-RGC axons grew beyond their future targets before innervating them. On E15, the axons of Cdh3-RGCs were seen in the optic tract, adjacent to the vLGN, IGL, and dLGN, but never within any of those targets (Figure 2A). At the same age, however, Cdh3-RGC axons were observed in the most distal visual target, the superior colliculus (SC; Figure 2B). Cdh3-RGC axons gradually increased in density within the SC until birth (Figures 2C, 2D, and 1Q), coinciding with innervation of thalamic and pretectal targets (Figure 1). They regressed from the SC after P0 such that by P20, only the occasional GFP-expressing axon was observed there (Figures 2D–2F and 1Q). Interestingly, the period when Cdh3-RGC axons were removed from the SC (P0–P20) coincided with the period when these axons increased in density within their more proximal targets, the OPN and PPN (Figure 1).

Removal of Cdh3-RGC Axons from the SC by Axon Retraction and Cell Death
To confirm the timing of Cdh3-RGC axon targeting, we carried out retrograde labeling (Figures 2G–2J). We injected cholera toxin β conjugated to Alexa Fluor 594 (CTβ–594) into the SC of P2 mice, then measured the percentage of CTβ+/Cdh3-GFP+ RGCs in the retina one day later on P3 (the "P2/3 group"; Figure 2J–L). We injected other mice on P19 and harvested their retinas on P20 (the "P19/20 group"; Figures 2M–2O). Approximately 29% of Cdh3-RGCs projected to the SC at P3 (Figures 2J–2L), whereas ~7% of Cdh3-RGCs projected to the SC at P20 (Figures 2M–20 and 2S). These values are generally consistent with our observations of Cdh3-RGC axons (Figures 2D and 2F).

To determine whether the removal of Cdh3-RGC axons from the SC reflects axon pruning, developmental cell death, or both, we injected the SC of P2 mice with CTβ–594 and waited until P20 to examine their retinas (the "P2/20 group"; Figures 2P–2R). By comparing the percentage of double-labeled CTβ+/Cdh3-GFP+ RGCs in the P2/3 versus P2/20 groups, we determined whether the RGCs that projected to the SC early in development remained viable. If both groups had the same percentage of double-labeled RGCs, we would conclude their axons retracted from the SC and either redirected or maintained...
Figure 1.2: Early ingrowth and subsequent removal of Cdh3-RGC axons to the superior colliculus (legend on next page)
their inputs to more proximal targets (e.g., vLGN, ONP, or PPN). In P2/20 mice, ~17% of Cdh3-RGCs were double labeled (Figures 2P–2S), which is ~60% of the double-labeled cells observed in the P2/3 group. Thus, of the Cdh3-RGCs that projected to the SC on P2, some must have retracted their axons from the target and survived. However, because ~40% of Cdh3-RGCs that projected to the SC on P2 were gone by P20, they were likely removed by cell death, which occurs in the first postnatal week (O’Leary et al., 1986; Farah and Easter, 2005). Indeed, the number of GFP-expressing Cdh3-RGCs was reduced by approximately half from P0 to P8 but remained consistent from P8 to P20 (Figure 2T). We therefore conclude that Cdh3-RGCs removed their inputs to the SC through a combination of axon retraction and cell death.

### Refinement of Cdh3-RGC Axons Is Unlikely to Reflect Dynamic Patterns of GFP Expression

The data presented thus far support the idea that Cdh3-RGCs undergo substantial overshoot and remodeling of their axon projections to achieve target specificity. To address if these changes simply reflect transient GFP expression, we analyzed which subtypes express Cdh3-GFP across development. We used three standard criteria for “typing” RGCs: dendritic morphology, dendritic laminar architecture, and cell-type-specific markers. First, we targeted live Cdh3-RGCs in explants and filled them to reveal their dendritic branching (Figure S3A). The same two morphologically distinct RGC subtypes expressed GFP in both adult (P28–P94) and P8 Cdh3-GFP mice: Cdh3-subtype 1, which was studded with spiny protrusions on its proximal and distal dendrites (Figures S3B – S3C), and Cdh3-subtype 2, which had a smaller dendritic tree with smooth proximal dendrites and fine-studded, distal dendrites (Figures S3D – S3E; n = 9 cells per age).

We also examined the dendritic stratification patterns of the Cdh3-RGCs in the retinas of P3, P8, and P20 mice costained for vesicle acetylcholine transporter (VAChT), which labels S2 and S4, the “OFF” and “ON” sublayers in the inner plexiform layer (IPL), respectively. At all ages, we found two consistent RGC subtype-specific patterns of dendritic stratification. Cdh3-subtype 1 monostratified its dendrites vitreal to S4 (Figures S3F – S3H), and Cdh3-subtype 2 bistratified its dendrites scleral to S2 and vitreal to S4 (Figures S3I – S3K).

Next, we stained Cdh3-GFP retinas for molecular markers known to restrict their expression to specific RGC types. Because early in development Cdh3-RGCs project to the dLGN and the SC we used two markers (Cart and SMI32) that, at maturity, label the RGCs that stably project to those targets (Dhande et al., 2013; Kay et al., 2011). At P8 and P20, ~8% of Cdh3-RGCs express Cart or SMI32 (Figures S3L – S3R). We also stained for melanopsin, which labels ~10% of adult Cdh3-RGCs (Osterhout et al., 2011). At P8, ~6% of Cdh3-RGCs expressed melanopsin, and this increased to ~11% by P20 (Figures S3L and S3S – S3X). The fact that the dendritic morphology, stratification, and molecular marker expression of Cdh3-RGCs is consistent from early postnatal development into adulthood argues that the changes in axon projection patterns we observed are unlikely to originate from shifting patterns of GFP expression but instead are likely to reflect reordering of axons projection patterns originating from the same two RGC subtypes across time.

### Highly Precise Axon Target Matching by Hoxd10-RGCs to the Accessory Optic System Occurs Postnatally

Next, we explored the emergence of axon target matching for Hoxd10-RGCs, which, at maturity, project mainly to nuclei of the accessory optic system (AOS; Figures S1H–S1K and S2G–S2L; Dhande et al., 2013). Hoxd10-RGC axons were first detectable in AOS targets, the medial terminal nucleus (MTN) and nucleus of the optic tract (NOT) at P7, their density increasing there from P8 to P15 (Figures 3A–3H). Hoxd10-RGC projections to their other targets, the dLGN and SC, also developed postnatally from P8 to P15 (Figures 3I–3N). Notably, we never observed Hoxd10-RGC axons innervating brain targets other than the ones they project to in the adult. Thus, Hoxd10-RGC axons arrive in the brain much later than Cdh3-RGC axons do and do not transiently sample any targets.

We considered the possibility that the apparent late arrival of Hoxd10-RGC axons reflected limited GFP expression at young ages. However, Hoxd10-RGCs expressed GFP as early as E16, and GFP+ somas were clearly visible throughout the ganglion cell layer of the retina starting at P0 (Figures S4F–S4J). We also used retrograde filling from the SC to address whether Hoxd10-RGCs project into the visual system at ages before P7. At P3, 19% of Hoxd10-RGCs projected to the SC.
Figure 3. Axon Target Matching for Hoxd10-RGCs Begins Postnatally

(A–D) Hoxd10-RGC axons in the dorsal and ventral medial terminal nucleus (dMTN and vMTN) at P7 (A), P8 (B), P15 (C), and P20 (D). Asterisk, nonretinal GFP-expressing neuron. Scale bar represents 250 μm.

(E–H) Hoxd10-RGC axons in the nucleus of the optic tract (NOT) at P7 (E), P8 (F), P15 (G), and P20 (H). Arrow in (F), cluster of GFP expressing axons. Scale bar in (I–K) represents 250 μm. Scale bar in (L–N) represents 40 μm.

(I–N) Hoxd10-RGC axons in SC at P8 (I), P15 (J), and P20 (K). Scale bar represents 250 μm.

(O–Q) Hoxd10-GFP retinas retrogradely labeled with CTβ-594 (O) from the SC on P2 followed by harvest and analysis of Hoxd10-RGCs (P) on P3. Circles, Hoxd10-RGCs that do not contain CTβ-594. Scale bar represents 100 μm.

(R–U) CTβ-594 (R) after injection to the SC at P16 (harvest on P17). Hoxd10-RGCs (S); (T) merge. Circles, GFP+ RGC somas that lack CTβ-594. Arrowheads, double-labeled RGCs. (U) Percentage of total GFP RGCs that are double-labeled (±SEM), n = 2 mice. *p < 0.05, **p < 0.01.

GFP in both young and mature Hoxd10-GFP mice. Nonetheless, we characterized the dendritic morphology and stratification patterns of Hoxd10-RGCs at P5, P8, and adult. At all ages, we observed the same two GFP-expressing RGC subtypes: the dendrites of Hoxd10-subtype 1 stratified in the S4 sublaminae of the IPL and had swellings on their distal processes (Figures S5A–S5D; Dhande et al., 2013). Thus, throughout the axon targeting phase, the same two subtypes of RGCs express GFP in this mouse line.

DRD4-RGC Axons Innervate Their Brain Targets Postnatally and Make Minimal Errors

We then analyzed axon target matching for a third population of RGCs, DRD4-RGCs, which at maturity project to the dLGN and SC, but not to the pretectum or to accessory optic targets (Figures S1H–S1M and S2M–S2R; Huberman et al., 2009; Kay et al., 2011). Although P0 DRD4-RGCs clearly expressed GFP (Figure S4L), very few DRD4-RGC axons were visible in the dLGN at that age (Figure 4A). Starting at P3, DRD4-RGC axons were visible in the optic tract and innervating the dLGN (Figure 4B). By P5, DRD4 axons were seen in the dLGN region adjacent to the optic tract.
Figure 1.4: DRD4-RGCs innervate their targets postnatally and transiently innervate one target (legend on next page).

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**DRD4-GFP**

**Visual thalamus**

**Superior colliculus**

**Central pretectum**

---

RGCs retrogradely labeled with CTβ-594 from Superior colliculus

<table>
<thead>
<tr>
<th>CTβ-594</th>
<th>DRD4-GFP</th>
<th>Merged</th>
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</thead>
<tbody>
<tr>
<td>P1</td>
<td><img src="image1" alt="" /></td>
<td><img src="image2" alt="" /></td>
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<tr>
<td>P5</td>
<td><img src="image3" alt="" /></td>
<td><img src="image4" alt="" /></td>
</tr>
</tbody>
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**P**

Maximal distance of GFP+ axons from OT (μm)

**Q**

% dGN with co-labeled CTβ-594+

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**Legend**

- dGN: deep ganglion
- CTβ-594: retrograde tracer
- DRD4-GFP: fluorescent protein
- NOT: nucleus of the optic tract
- OT: optic tract

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called the “shell” (Figure 4C; Krahe et al., 2011), their density increasing there from P5 to P20 (Figures 4C-4E and 4P). Interestingly, when they first arrived, some DRD4-RGC axons overshot the shell into the more medial dLGN and then refined to the correct zone (Figure 4C, inset, and quantified in Figure 4P).

DRD4-RGC projections to the SC followed a similar overall time course: their axons arrived from P0 to P5 (Figures 4F and 4G), filled the upper stratum griseum superficiale (uSGS) by P8, and appeared adult-like by P20 (Figure 4H, I). We confirmed this timeline by retrograde labeling and found that 23% of DRD4-RGCs projected to the SC on P1, ~42% on P3, and ~93% on P5 (Figures 4J-4Q and 4Q). Thus, just like Hoxd10-RGC axons, DRD4-RGC axons innervate their targets postnatally.

**DRD4-RGCs Transiently Project to the Accessory Optic System**

In contrast to Hoxd10-RGCs, we found that DRD4-RGCs initially trespassed into an accessory optic target; they innervated the NOT and persisted there from P5 to P8 (Figures 4R and 4S), after which they exited this target between P15 and P20 (Figures 4T and 4U). Interestingly, the timing of DRD4-RGC axon removal from the NOT coincided with the stage when Hoxd10-RGC axons entered this target (Figure 3F), suggesting possible competition between axons arising from these two RGC populations (see discussion below).

To address the possibility that DRD4-RGC projections to the NOT resulted from transient expression of GFP in AOS-projecting RGC subtypes, we examined their morphologies and dendritic lamination patterns across development. At P8 and P20, DRD4-RGCs displayed the classically described dendritic characteristics of On-Off DSGCs (Figures S5A-S5J). In addition, the dendrites of P3 DRD4-RGCs bistriated and cofasciculated with the processes of starburst amacrine cells in S2 and S4 of the IPL, hallmark features of On-Off DSGCs (Figures S5K-S5P; Huberman et al., 2009; Beier et al., 2013). At both P8 and P20, ~100% of DRD4-RGCs expressed the On-Off DSGC marker, Cart (Kay et al., 2011), and < 1% expressed the alpha RGC marker, SMI32 (Figures S5Q-S5Y). Thus, we conclude that transient DRD4-RGC axon projections observed in the NOT arise from genuine mistargeting of these axons to the AOS rather than transient expression of GFP in other RGC subtypes.

**Other Late-Arriving RGC Populations Are Also Highly Accurate in Their Targeting Choices**

To further test whether the time of axon arrival relates to axon target matching strategy, we examined a fourth mouse line, TRHR-GFP, which labels On-Off DSGCs with distinct physiological characteristics and overall projection patterns from that of DRD4-RGCs, Cdh3-RGCs, or Hoxd10-RGCs (Figure S2; Rivlin-Etzion et al., 2011; Stafford et al., 2014). TRHR-RGCs arrived in the brain and innervated their targets predominantly during the second postnatal week (Figures 5C, 5G, and 5I). Few TRHR-RGC axons were visible in the dLGN or SC at P5 (Figures 5A and 5E) but from P8-P20 they filled the dLGN shell (Figures 5B-5D) and innervated the uSGS of the SC by P8 (Figures 5F-5H). TRHR-RGC axonal projections to the NOT followed a similar time course (Figure 5I). We did not observe evidence for TRHR-RGCs transiently innervating any targets (Figure 5I). Thus TRHR-RGCs, just like Hoxd10-RGCs, arrive in the brain relatively late and select their correct targets from the outset. When compared to each other, the four RGC populations examined here reveal a clear relationship between the time of axon arrival and the number of incorrect targets transiently innervated (Figure 5J).

**Birthdates Vary across RGC Subtypes in a Manner that Correlates with Target Innervation Strategy**

If the time when an RGC axon arrives in the visual pathway is an important determinant of the target-matching strategy it uses, then it is important to consider the factors that underlie that timing. One idea is timing of birth. In order to address whether the different RGC populations examined are born at different stages, we labeled terminally dividing cells with 5-ethynyl-2'-deoxyuridine (EdU) in Cdh3-, DRD4-, and Hoxd10-GFP mice during embryogenesis. We injected pregnant female mice with EdU at E10, E12, E14, or E16, harvested their offspring’s retinas when they were P8, and quantified the number of EdU/GFP+ RGCs (Voinescu et al., 2009; McNeill et al., 2011). We discovered that each of the three RGC populations had unique birthdating profiles (Figures 5K-5U). Approximately 25% of Cdh3-RGCs were born at E10, and the number of newly born Cdh3-RGCs peaked at E12 (Figures 5K-5M and 5T). By contrast, only ~5% of DRD4-RGCs were born at E10, and the number of newly born EdU-labeled DRD4-RGCs peaked at E14 (Figures 5N-5P and 5T). The fraction of EdU-labeled Hoxd10-RGCs was also highest at E14, but a higher fraction of them were born at E16 relative to the other two RGC populations we examined (Figure 5T).
To account for any regional variations across the retina in RGC birthdating profiles, we also examined when 50% of EdU/GFP double-labeled RGCs were born in the central versus peripheral retina. Although there were slight differences according to retinal location (Figure 5U), this did not alter the overall relationship between RGC population and birthdate described above; neurogenesis of Cdh3-RGCs occurs relatively earlier than for other RGC populations. DRD4- and Hoxd10-RGCs were born at similar times, with Hoxd10-RGCs exhibiting a relatively protracted period of neurogenesis (Figures 5T and 5U). Along with
the findings above, these results indicate that RGC-category-specific birthdate correlates with when and how RGC axons select their targets.

**DISCUSSION**

Here, we explored how functionally distinct retinal neurons connect to their appropriate targets in the brain. Cellular explorations of other visual circuit assembly events have been instrumental in defining molecular models of those processes and indeed went on to become broadly influential. For example, models of retinotopic and eye-specific mapping emerged from the findings that RGCs initially overshoot their correct topographic and eye-specific zones before remapping to the appropriate locations, examples that thematically extend across many brain circuits (Shatz, 1996; McLaughlin and O’Leary, 2005; Feldheim and O’Leary, 2010).

Our first discovery is that different RGC populations employ different strategies to achieve accurate axon target matching. Cdh3-RGCs extended the entire length of the visual pathway before innervating intermediate targets. Subsequently, these axons refined their projections, stabilizing only those located in correct targets. The finding that RGCs sample different targets before refining their connections has precedence from classic studies in other species (Ramoa et al., 1989) but whether this was a general rule for all RGCs was not addressed. In fact, transient sampling of incorrect targets is not a general rule. The axons of Hoxd10-, DRD4-, and TRHR-RGCs were far more selective. DRD4-RGCs transiently sampled only one target (the NOT), and both Hoxd10- and TRHR-RGCs exhibited no transient targeting whatsoever.

Our second finding is that strategy of axon target matching is correlated with timing of axon growth. The axons of the three populations of RGCs that underwent minimal target sampling all arrived at their targets postnatally. By contrast, Cdh3-RGC axons reached the brain very early and underwent widespread refinement. Type 1 intrinsically photosensitive RGCs (M1 ipRGCs) also innervate their targets postnatally and do not make targeting errors (McNeill et al., 2011; Su et al., 2011). Thus, for five parallel eye-to-brain pathways, time of axon arrival correlates with the frequency of transient target innervation; early arrival correlates with extensive transient targeting, late arrival leads to no transient targeting, and axons that arrive in the interim transiently sample a minimal number of targets (Figure 5J).

Third, we found that RGC birthdates systematically relate to targeting strategy; early-born RGCs undergo extensive readjusting of their initial targeting choices compared to later-born RGCs. The impact of birthdate on targeting strategy may ultimately relate to differences in axon growth rates. Although RGCs are born during a relatively narrow timeframe (Figure 5T), they innervate the brain across a relatively broad period spanning pre- and postnatal life. RGC axons are known to undergo a dramatic reduction in growth rate as they transition from embryonic to postnatal period (Goldberg et al., 2002). Given our observations that Cdh3-, Hoxd10-, and DRD4-RGCs exhibit different birthdate profiles, they likely possess different axon growth rates as well.

**Similarities between Axon Target Matching, Retinotopic, and Eye-Specific Mapping**

The overshoot and transient sampling of intermediate targets in Cdh3-RGCs is reminiscent of topographic mapping, whereby RGC axons initially extend across the full extent of the SC and then remove the overshooting portion of their axon, a process that involves axon-axon competition (McLaughlin and O’Leary, 2005; Feldheim and O’Leary, 2010). Axon target matching may also involve competition, with early-arriving RGCs limiting target vacancy and thereby preventing entrance of subsequent-arriving axons. An “early arrival” competition model has also been proposed to explain development of eye-specific layers (Shatz, 1996). Future studies involving selective deletion of early-arriving RGCs would help test whether competitive interactions indeed regulate axon target matching.

**Molecular Mechanisms for Axon Target Matching**

RGC axon target matching very likely relies on both repellants and attractants. All populations of RGCs we examined grew past the suprachiasmatic nucleus, suggesting this target harbors repellants for many non-M1 RGCs. Silt-robo-repellant interactions prevent mammalian RGCs from growing into the ventral diencephalon (Ringstedt et al., 2009); such repulsion may act at various locations and spatial scales to influence RGC axon target specificity. Adhesion also plays a role in axon target matching. Cadherin-6 (Cdh6) is expressed by Cdh3-RGCs and by their targets. In mice lacking Cdh6, Cdh3-RGCs incorrectly project beyond those targets (Osterhout et al., 2011). In addition, Reelin (which can regulate cadherin expression) is required for accurate ipRGC targeting in the thalamus (Franco et al., 2011; Su et al., 2011).

**Varying Modes of Axon Target Matching and the Establishment of Polysynaptic Circuits**

An important consideration is that cells within brain targets are undergoing maturation during the same stages when axons innervate them. Generally speaking, early-arriving axons may play an important role in the maturation of targets and/or prime growth pathways for the arrival of subsequent axons via expression of molecular signals. Indeed, Chen and coworkers described a critical role for early-arriving “pioneer” axons in targeting of subsequent-arriving axons to the zebrafish tectum (Pittman et al., 2009). Notably, the arrival of RGC axons in the dLGN regulates ingrowth timing of corticogeniculate afferents by influencing repellent expression (Brooks et al., 2013; Seabrook et al., 2013). Thus, whether or not an axon chooses to bypass, transiently innervate, or stably connect to a given target may instruct the maturation of that immediate target and its downstream targets that together comprise parallel pathways. In these ways, the variety of targeting strategies used by functionally distinct neurons such as those demonstrated here could have broad influence on the overall wiring specificity of circuits in the mammalian brain.

**EXPERIMENTAL PROCEDURES**

**Animals**

Cadherin-3-EGFP (Cdh3-GFP), homeobox D10 enhanced GFP (EGRF; Hoxd10-GFP), dopamine receptor D4-EGFP (DRD4-GFP), and thyrotropin-releasing hormone receptor-EGFP (TRHR-GFP) mice were obtained.
from MMRRC (Huberman et al., 2009; Osterhout et al., 2011; Rivlin-Etzion et al., 2011; Dhande et al., 2013). All procedures were in accordance with institutionally approved protocols at the University of California, San Diego.

**Tissue Processing**

Tissue was immunostained to enhance the GFP signal (Huberman et al., 2008). Antibodies used were rabbit anti-GFP (1:1,000; Invitrogen), guinea pig anti-GFP (1:1,000; Syaptic Systems), guinea pig anti-VAChT (1:1,000; Millipore), rabbit anti-Cart (1:1,000; Phoenix), mouse anti-SMI32 (1:1,000, Invitrogen), and rabbit anti-melanopsin (1:1,000; Advanced Targeting Systems).

**Anterograde and Retrograde Labeling of RGC Axons**

Anterograde labeling with CTb-594 was as described previously (Huberman et al., 2008). For retrograde labeling, a pulled-glass capillary pipette was used to inject through the skull; CTb-594 (0.5–1.0 μl at 0.5% in saline) was bilaterally pressure-injected into the SC.

**Identifying Targets and Within-Target Locations for Analysis**

Retinorecipient nuclei were identified by whole-eye labeling with CTb-594 and target boundaries determined by landmarks and comparison to Godement et al. (1984). Images were acquired from the middle third of each target.

**Quantification of Percentage of Target Area Occupied by GFP-Expressing Axons**

Area fraction measurements were quantified in ImageJ. Mean pixel value of the background was measured in a 250 μm × 250 μm area devoid of GFP labeling and then used to subtract background signal. The "area fraction" tool was used to find the percent of target occupied by GFP+ axons. Measurements were taken from three to eight tissue sections in each target, depending on target size and age.

**Quantification of Within-dLGN Refinement**

Maximum distance occupied by axons across the lateral-medial extent of the dLGN taken with the "line measurement" tool in ImageJ. Three measurements per tissue section (dorsal, middle, and ventral) and three tissue sections per animal (rostral, middle, and caudal) were analyzed, for a total of nine measurements per mouse (n = 5 mice per age group).

**RGC Marker Analysis**

Retinas from three mice were analyzed. Approximately 100 GFP+ RGCs, from multiple 250 μm × 250 μm regions at varying distances from the optic nerve head, were analyzed.

**Targeted RGC Injections/Filling**

Targeted fills were carried out as described previously (Beier et al., 2013).

**Birthing RGCs**

Pregnant females were injected with EdU (20 μg/g body weight; Invitrogen). The Click-IT EdU Alexa Fluor Imaging kit (Invitrogen) was used to detect EdU before primary antibody staining. EdU-labeled RGCs were quantified from eight to ten retinal sections (n = 3). A cumulative fraction graph using a two-degree polynomial curve was used to calculate the day at which 50% of cells were born in the central and peripheral retina (R² > 0.98 for all curves; Voinescu et al., 2009).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.063.

**AUTHOR CONTRIBUTIONS**

J.A.O. and A.D.H. conceived the experiments, J.A.O. and P.L.N. performed histology, J.A.O. imaged and analyzed all data, R.N.E.-D. did the RGC filling, and J.A.O. and A.D.H. wrote the paper.

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**REFERENCES**


CHAPTER 1 SUPPLEMENTAL DATA

Figure 1.S1: Axon target matching and transgenic tools for labeling specific RGC subtypes in the mammalian visual system
Figure S1. (Related to Figures 1-5) Axon-target matching and transgenic tools for labeling specific RGC subtypes in the mammalian visual system.

**A** Axon-target matching involves functionally distinct subtypes of neurons whose cell bodies reside in one structure, and whose axons project to different targets in the brain. These axons pathfind together and at some point, make the decision to depart their path and innervate specific targets. **B** Possible scenarios for axon-target matching. **C-D** CTβ-594 labeling of RGC axons shown in grayscale (C) and inverted grayscale (D). **E-F** Dorsal and ventral view of the brain from CTβ-594 injected mouse. SC: superior colliculus. Ctx: cortex. ON: optic nerve. OC: optic chiasm. OT: optic tract. SCN: suprachiasmatic nucleus. MTN: medial terminal nucleus. r: rostral; c: caudal. Numbering represents the locations from which tissue sections were taken in panels J-M below. Scale = 2.5mm. **G** Sagittal view of the brain with one cortical hemisphere. dLG: dorsal lateral geniculate nucleus. vLG: ventral lateral geniculate nucleus. NOT: nucleus of the optic tract. PPN: posterior pretectal nucleus. d: dorsal; v: ventral. Scale = 5mm. **H** A schematic of the retina; PRL: photoreceptor layer. opl: outer plexiform layer; INL: inner nuclear layer. ipl: inner plexiform layer with on and off sublaminar zones; GCL: ganglion cell layer where the cell bodies of RGCs reside. **I-M** Distinct groups of RGC subtypes and their central targets color coded: Cdh3-RGCs and targets (orange), Hoxd10-RGCs and targets (magenta), DRD4-RGCs and targets (blue), TRHR-RGCs and targets (yellow). **I** The RGC subtypes and their characteristic dendritic stratification patterns. **J-M** Schematics of coronal brain sections corresponding to the locations (#1-4) in the brain shown in panels E and F. Color coding is used to show which targets receive projections from the RGC subtypes summarized in panel (I). OPN: olivary pretectal nucleus.
Figure 1.S2: Axon target matching of Cdh3- Hoxd10- DRD4- and TRHR-RGCs is complete by P20
Figure S2. (Related to Figures 1-5) Axon-target matching of Cdh3- Hoxd10- DRD4- and TRHR-RGCs is complete by P20.

(A-X) CTβ-594 (red) labeled RGC axons identified the boarders of retinorecipient targets. All scales = 250μm. (A-F') Projection pattern of Cdh3-GFP RGCs (Cdh3-RGCs) at P20. Cdh3-RGCs project to the vLGN (B, B'; arrow) the OPN (C, C'), and to the PPN in the pretectum (D, D'). Cdh3-RGCs do not project to the SCN (A, A'; arrowhead: Cdh3-RGC axon in the optic chiasm (OC); asterisk: GFP expressing radial glia in the third ventricle) to the dLGN (B, B'; arrowheads indicate sparse fibers), to the MTN (E, E') or the SC (F, F'). IGL; intergeniculate leaflet. uSGS: upper stratum griseum superficiale. ISGS: lower stratum griseum superficiale. (G-L') Projection pattern of Hoxd10-GFP RGCs (Hoxd10-RGCs) at P20. Hoxd10-RGCs innervate the lateral shell of the dLGN (H, H'; arrowheads), the NOT (J, J'; asterisks indicate surrounding central pretectal nuclei), the MTN (K, K') and the uSGS in the SC (L, L'; arrowheads). (G, G') Hoxd10-RGCs axons do not project to the SCN (G, G'; arrowheads show Hoxd10-RGC axons travelling between the two SCN towards the accessory optic tract), or the OPN (I, I'). MTNv: ventral division of the medial terminal nucleus. MTNd: dorsal division of the medial terminal nucleus (MTNd). (M-R') Projection pattern of DRD4-GFP RGC (DRD4-RGCs) at P20. DRD4-RGCs project to the dorsal shell of the dLGN (N, N'; arrowheads; asterisks indicate sparse projections to the vLGN), and the uSGS of the SC (R, R'). They do not project to the SCN (M, M'), the OPN (O, O'), the NOT (P, P') or the MTN (Q, Q'; asterisk denotes GFP-expressing axons of non-retinal origin in non-visual areas of the brainstem surrounding MTN). (S-X') Projection pattern of TRHR-GFP RGCs (TRHR-RGCs) at P20. TRHR-RGCs do not innervation the SCN (S, S'), the OPN (U, U') or the MTN (W, W'). Asterisks indicate cell bodies or processes of GFP expressing cells that originate in the brain. TRHR-RGCs project to the dorsal shell of the dLGN and the vLGN (T, T'), a subregion of the NOT (V, V') and the upper SGS of the SC (X, X').
Figure 1.S3: Cdh3-GFP mice express GFP in the same RGCs in early and late development.
Figure S3. (Related to Figures 1 and 2) Cdh3-GFP mouse expresses GFP in the same RGCs in early and late development.

(A-E) Targeted intracellular filling of Cdh3-RGCs (n= 9 cells filled per age). Scale = 50μm for all panels. (A) GFP expressing cells (red arrowheads) were targeted and filled with Alexa dye (B-C'') Targeted fills of Cdh3-subtype 1 found in adult (P28-P94; B) and P8 mice (C). High magnification views of subtype 1 show spiny protrusions on the proximal (B’, C’; red arrowheads) and distal dendrites (B”’, C”’; black arrows). (D-E’’) Targeted fills of Cdh3-subtype 2 in adult (D) and P8 (E) mice. High magnification views of subtype 2 shows swellings along the proximal (D’, E’; red arrowheads) and distal (D”’, E”’; black arrowheads) dendrites. (F-K’) Sections of Cdh3-GFP retinas at P3 (F, I), P8 (G, J), and P20 (H, K); subtype 1 (F-H’) and subtype 2 (I-K’). F’-K’ show high magnification views (arrows indicate Cdh3-RGC dendrites). Scale for F-K = 100μm. (L) Quantification of cell-type markers expressed by Cdh3-RGCs at P8 and P20 (±SEM); 80-100 RGCs quantified per mouse; n= 3 mice for each marker, per age). (M-R) Whole mount Cdh3-GFP retinas co-stained with GFP (green) Cart (magenta) and SMI32 (cyan) at P8 (M-O) and P20 (P-R). Circles indicate Cdh3-RGCs that do not express Cart or SMI32 at P8 or P20. (S-X) Cdh3-GFP retinas co-stained with GFP (green) and melanopsin (magenta) at P8 (S-U) and P20 (V-X). Arrowheads indicate Cdh3-RGCs that also express melanopsin at P8 and P20. Scale = 100μm.
Figure 1.S4: Cdh3- Hoxd10- and DRD4-RGCs begin expressing GFP before birth
Figure S4. (Related to Figures 1-5) Cdh3-, Hoxd10-, and DRD4-RGCs begin expressing GFP before birth.

The first column is a retina section at E16 and the remaining columns are images of whole mount retinas. Scales in E16 sections = 250µm. Scales for remaining panels = 50µm. (A-E) GFP expression in Cdh3-RGCs at E16 (A), P0 (B), P3 (C), P8 (D), and P20 (E). (F-J) GFP expression in Hoxd10-RGCs at E16 (F; arrowhead points to a migrating GFP-expressing RGC also shown at high magnification in F’), P0 (G), P3 (H), P8 (I), and P20 (J). (K-O) GFP expression in DRD4-RGCs at E16 (K), P0 (L), P3 (M), P8 (N), and P20 (O).
Figure 1.S5: Hoxd10- and DRD4-GFP mice express GFP in the same RGCs during early and late development.
Figure S5. (Related to Figures 3 and 4) Hoxd10- and DRD4-GFP mice express GFP in the same RGCs during early and late development.

(A-H) Identification of Hoxd10-RGCs by targeted intracellular injections in adult (P25-45) and P8 mice (n= 10 cells filled per group) and their dendritic lamination patterns P5 and P20 mice. A, B, E, F Scale = 50µm. (A-B) Hoxd10-subtype 1 in adult (A, A’) and P8 (B, B’) Hoxd10-GFP mice. Arrowheads in A’ and B’ indicate distal swellings. (C-D) Dendritic lamination of Hoxd10-subtype 1 at P5 (C) and P20 (D); high magnification in C’ and D’; scale =100µm. (E-F) Hoxd10-subtype 2 in adult (E, E’) and P8 (F, F’) mice, scale = 50µm. Arrowheads in E’ and F’ indicate end-terminal swellings. (G-H) Dendritic lamination of Hoxd10-subtype 2 at P5 (G) and P20 (H); high magnification views in G’ and H’; arrowheads indicate Hoxd10-RGC dendrites; arrow indicates dendrites traversing from S4 to S2; scale =100µm. (I-J) Targeted intracellular fills of DRD4-RGCs revealed one morphological subtype at P25 (I) and P8 (J; n= 11 RGCs filled per age). Arrowheads in I’ and J’ indicate thick primary dendrites. Arrowheads in I’’ and J’’ indicate looping arborizations on distal. Scale = 50µm. (K-P) Retina sections stained for GFP (Green), VACHT (magenta) and DAPI (cyan) at P3 (K-L’), P8 (M-N’) and P20 (O-P’); arrowheads indicate DRD4-RGC dendrites that co-fasciculated with S4 and S2 VACHT-expressing amacrine processes. Scale = 100µm. (K’, L’, M’, N’, O’, P’) High magnification views of DRD4-RGC dendritic processes. (Q-Y) Molecular markers expressed by DRD4-RGCs (green). Cart (magenta, which labels On-Off DSGCs: Kay et al., 2011) and SMI32 (cyan) labeling at P8 (Q-T) and P20 (U-X). (S); Merge shown in T. (U-X) The same was true at P20; DRD4-RGCs (arrows; U) always co-expressed Cart (V) but not SMI32 (W); Merge shown in X. Scale = 100µm. (Y) Quantification of the fraction of GFP expressing RGCs that also expressed Cart or SMI32 (±SEM). GFP/Cart: P8= 94.56%, P20= 96.32%; GFP/SMI32’ P8= 0.26%, P20= 0%. Number of cells counted per mouse = >100; n= 3 mice analyzed per age/marker.
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CHAPTER 2: Cadherin-6 mediates axon-target matching in a non-image-forming visual circuit

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Cadherin-6 Mediates Axon-Target Matching in a Non-Image-Forming Visual Circuit

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SUMMARY

Neural circuits consist of highly precise connections among specific types of neurons that serve a common functional goal. How neurons distinguish among different synaptic targets to form functionally precise circuits remains largely unknown. Here, we show that during development, the adhesion molecule cadherin-6 (Cdh6) is expressed by a subset of retinal ganglion cells (RGCs) and also by their targets in the brain. All of the Cdh6-expressing retinorecipient nuclei mediate non-image-forming visual functions. A screen of mice expressing GFP in specific subsets of RGCs revealed that Cdh3-RGCs which also express Cdh6 selectively innervate Cdh6-expressing retinorecipient targets. Moreover, in Cdh6-deficient mice, the axons of Cdh3-RGCs fail to properly innervate their targets and instead project to other visual nuclei. These findings provide functional evidence that classical cadherins promote mammalian CNS circuit development by ensuring that axons of specific cell types connect to their appropriate synaptic targets.

INTRODUCTION

Functional neural circuits consist of precise connectivity between specific sets of neurons. The assembly of such circuitry often requires that axons bypass numerous targets before selectively terminating in just one or a few specific targets. Over the last century, much progress has been made in understanding how axons undergo directed growth and pathfinding and how they form topographic maps (Sperry, 1963; Dickson, 2002; Feldheim and O’Leary, 2010). How mammalian axons identify which targets to innervate, however, remains poorly understood.

The axonal connections formed by the eyes with the brain are an attractive model for exploring mechanisms of axon-target recognition in the mammalian CNS. Retinal ganglion cells (RGCs) are the output neurons of the eye and they are divided into ~20 different types. Each RGC type encodes a different quality of the visual scene, such as brightness, direction of motion or edges (Masland, 2001; Berson, 2008), and sends that information to a limited number of retinorecipient targets that in turn regulate specific aspects of perception and behavior. For example, type 1 melanopsin RGCs encode the level of environmental illumination and project to the hypothalamic suprachiasmatic nucleus and midbrain olivary pretectal nuclei, which are involved, respectively, in entrainment of circadian rhythms and pupillary reflexes (Ecker et al., 2010; Güler et al., 2008). In contrast, the axons of alpha and On-Off direction selective RGCs innervate the dorsal lateral geniculate nucleus (dLGN) and the superior colliculus (SC) (e.g., Bowling and Michael, 1980; Tamamaki et al., 1995; Huberman et al., 2008, 2009; Rivlin-Etzion et al., 2011), targets involved in pattern vision and visually guided gaze shifts.

What mechanisms enable CNS axons to connect to specific targets and to avoid others? In the developing Drosophila visual system, adhesion plays a critical role in axon-target matching (Clandinin and Feldheim 2009). The cadherins are a family of molecules hypothesized to establish precise CNS connectivity by promoting selective adhesion among neurons expressing the same cadherin or combination of cadherins (Takeichi, 2007). Previous work showed that N-Cadherin is important for targeting specificity of Drosophila photoreceptors: loss of function mutations and experiments with genetically mosaic animals demonstrated that N-cadherin is required both in photoreceptors R1-R6 and in their target lamina neurons (Lee et al., 2001; Prakash et al., 2005). In chick, antibodies against N-cadherin disrupt laminar specific RGC axon targeting in vitro (Inoue and Sanes, 1997). Whether cadherins regulate axon-target matching in the mammalian CNS, however, remains unknown.

Here, we show that Cadherin-6 (Cdh6) is expressed by a subset of RGCs and by their retinorecipient targets in the brain, all of which mediate non-image-forming visual functions. We also show that Cdh3-GFP and Cdh6-GFP transgenic mice label the RGCs that innervate Cdh6 expressing targets. We then provide...
Figure 2.1: Cadherin-6 is expressed in specific subcortical visual nuclei.

RESULTS

Cadherin-6 Is Expressed by a Subset of RGCs and Non-Image-Forming Visual Targets

As a first step toward assessing the role of cadherins in mammalian visual circuit assembly, we analyzed the expression patterns of several classical cadherins in the mouse brain. We visualized retinorecipient targets by making bilateral intravitreal injections of cholera toxin beta conjugated to Alexa 594 (CTb-594) which labels all RGC axons, and then compared each CTb-594 labeled target in the brain with the mRNA expression patterns of cadherin 1 (Cdh1), Cdh2, Cdh3, Cdh4, Cdh5, Cdh6, Cdh7, and Cdh8. We took particular notice of Cdh6 because it was highly and selectively expressed by a subset of the retinorecipient targets that mediate non-image-forming visual functions: the thalamic ventral lateral geniculate nucleus (vLGN), the intergeniculate leaflet (IGL) (Figures 1B and 1C), the olivary pretectal nuclei (OPN) (Figures 1D, 1E, and 1M) and the medial division of the posterior pretectal nucleus (mdPPN) (Figures 1F and 1G). The Cdh6-expressing targets relate to circadian rhythm entrainment (vLGN and IGL) (Harrington, 1997), pupil constriction (Gu¨ler et al., 2008) and oculomotor functions (mdPPN) (Giolli et al., 2006). Cdh6 expression was specific to these targets during late embryonic and early postnatal development (~E18–P4), the stage when RGC axons innervate their targets (Gode ment et al., 1984) with Cdh6 expression persisting into the first postnatal week (Figure 1). The other cadherins we assayed showed patterns of expression that were notably different from Cdh6. Cdh1, 3, 4, 5, 7, and 8 were not expressed by the OPN or mdPPN although Cdh4, 7, and 8 were expressed by other retinorecipient nuclei (Figures 1H, 1J, 1K, 1L, 1N, and 1O and unpublished observations). Indeed, Cdh4 and Cdh8 were expressed by regions adjacent to and surrounding the OPN but not in the OPN itself (asterisks). Cdh1 (H, 3 (J), 5 (L), and 7 (N) are not expressed by the OPN or nearby nuclei but some of these (e.g., Cdh7) are expressed by other retinorecipient targets (not shown). (H–O) Scale = 150 µm. See also Figure S1.

genetic evidence that deletion of Cdh6 causes defects in axon-target matching in this component of the retinofugal pathway.

Figure 1. Cadherin-6 is Expressed in Specific Subcortical Visual Nuclei
(A) CTb-594 labeled RGC axons at the forebrain-midbrain border of a postnatal day 2 (P2) mouse. Image is in coronal plane. Bracketed regions correspond to panels (B and D). Scale = 500 µm. (B) Pan-RGC axon labeling in the visual thalamus. The ventral lateral geniculate nucleus (vLGN), intergeniculate leaflet (IGL) and dorsal lateral geniculate nucleus (dLGN) contain RGC axons. (C) Cdh6 mRNA expressing cells in the IGL and vLGN. Scale = 100 µm. (D) CTb-594 labeled RGC axons in the rostral pretectum. The left and right olivary pretectal nuclei (OPN) are densely innervated. (E) Cdh6 mRNA expression in the OPN of a P1 mouse. (F) RGC axons in the caudal pretectum. The medial division of the posterior pretectal nucleus (mdPPN) of Scala (1972) appear as two foci (arrows). (G) Cdh6 mRNA in the mdPPN (arrows) of a P1 mouse. Asterisk: a few Cdh6 expressing cell; these may correspond to the caudal-most OPN. Scale = (D)–(G), 250 µm. (H–O) Cdh1–8 antisense mRNA labeling in the rostral pretectum of the early postnatal mouse. Cdh2 (I) and Cdh6 (M) are expressed by the OPN, whereas Cdh4 (K) and Cdh8 (O) are expressed by cells nearby the OPN but not in the OPN itself (asterisks). Cdh1 (H, 3 (J), 5 (L), and 7 (N) are not expressed by the OPN or nearby nuclei but some of these (e.g., Cdh7) are expressed by other retinorecipient targets (not shown). (H–O) Scale = 150 µm. See also Figure S1.
select their targets in the brain, the adhesion molecule Cdh6 is selectively expressed by a subset of non-image-forming retinorecipient targets.

**Cdh3-RGCs Selectively Innervate Cdh6-Expressing Visual Targets in the Brain**

To examine whether Cdh6 plays a functional role in retinofugal targeting, we needed a way to visualize the axons of the particular RGCs that innervate Cdh6 expressing visual targets. We screened a library of BAC transgenic mice \((Gong et al., 2003)\) and found that Cdh3-GFP mice selectively label the RGCs that innervate Cdh6 expressing targets (Figure 2 and see Figure S1 available online). We injected CTb-594 into both eyes of Cdh3-GFP mice (ages P0–P20) and then examined each of those targets for the axons of Cdh3-GFP RGCs (hereafter referred to as Cdh3-RGCs). Cdh3-RGC axons terminated in the vLGN and IGL, whereas the adjacent dLGN, the target that relays visual information to the cortex for image perception, was virtually devoid of Cdh3-RGC axons (Figures 2A–2E and S1). Cdh3-RGC axons also densely innervated the OPN (Figures 2A, 2B, 2F–2I, and S1) specifically in the OPN “core,” whereas the OPN “shell” was devoid of Cdh3-RGC axons (Figures 2H and 2I). A limited number of Cdh3-RGC axons remained in the optic tract until they arrived to the caudal pretectum, wherein they terminated in two dense foci corresponding to the mdPPN (Figures 2J and 2K; Scalia, 1972).

We are confident the GFP axons observed in the vLGN, IGL, OPN, and mdPPN originated from RGCs because they disappeared from those targets following eye removal (not shown). Indeed, with the exception of olfactory glia, a subset of brainstem nuclei and a small population of cells near the fourth ventricle, the brains of Cdh3-GFP mice were remarkably devoid of GFP-expressing cells (Figures 2A, 2B, S1, and S2). We found no evidence of Cdh3 mRNA expression in any retinorecipient targets (Figures 1J and S2). Together, our data indicate that Cdh3-GFP mice selectively label the RGCs that project to the vLGN, IGL, OPN, and mdPPN, the very same non-image-forming retinorecipient targets that express Cdh6.

**Cdh3-RGCs Include Several Different Types, All of Which Express Cdh6**

The limited number of retinorecipient targets innervated by Cdh3-RGCs prompted us to investigate which RGC types express GFP in this mouse line. Cdh3-RGCs represent \(~1\)% of...
Figure 2.3: Cdh3-GFP and Cdh6 expression in a subset of retinal ganglion cells

- **Figure 2.3 A**: GFP+ somas and axons in the ganglion cell layer (GCL) of a flat mounted Cdh3-GFP retina. Arrow: GFP+ axons.
- **Figure 2.3 B**: GFP+ somas and dendrites of Cdh3-RGCs. GFP+ amacrine cell somas (arrowheads) are also seen deep to the plane of focus. Scale = 200 μm (A) and 50 μm (B).
- **Figure 2.3 C**: Cdh3-RGC in a retinal section; the cell stratifies its dendrites proximal to the GCL, in the "On" sublamina of IPL. Dashed lines: IPL boundaries. Asterisk: a GFP+ amacrine. (D–F) Targeted intra-cellular injections with Neurobiotin (schematized in D; magenta label in E and F) reveal the morphology of individual Cdh3-RGCs. (D) Cdh3-RGCs without injection. (E) A Cdh3-RGC with a symmetric, sparsely branched dendritic arbor. (F) A different filled Cdh3-RGC. This cell has a densely branched, asymmetric arbor (F). Scale = 50 μm (D–F). (G–I) A subset of Cdh3-RGCs express melanopsin. (G) Cdh3-RGCs (open and solid arrowheads) and an amacrine (asterisk). (H) Two RGCs and their dendrites expressing melanopsin (red). (I) Merge of (G) and (H). One of the Cdh3-RGCs expresses melanopsin (solid arrowhead) whereas the other does not (open arrowhead), a non-GFP+ melanopsin RGC is also present (asterisk). Scale = 50 μm. (J and K) Confocal z stack of a Neurobiotin-filled Cdh3-RGC whose dendrites stratify mainly in the On-sublamina of the IPL. The cell extends a secondary dendritic arbor into the Off-sublamina (arrowhead). (K) ChAT stained amacrine cell bodies and dendrites (blue). Scale = 10 μm (J and K). (L and M) Cdh6 mRNA is expressed in the GCL (L) Cdh6 antisense. (M) Cdh6 sense control. Scale = 100 μm. (N–Q) Retinal section with (N) Dapi+ nuclei (blue), (O) Cdh3-RGCs, and (P) Cdh6 immunoreactive cells. (Q) Merge of (O) and (P). All Cdh6-RGCs express Cdh6 (arrowheads); but some Cdh6-immunopositive cells are not Cdh3-GFP+. Scale = 50 μm. See also Figure S3.

We next wanted to determine whether Cdh3-RGCs also express Cdh6. We found that Cdh6 mRNA was expressed by a subset of cells in the early postnatal RGC layer (Figures 3L and 3M), which is in agreement with a previous report (Honjo et al., 2000). Immunostaining revealed that all Cdh3-RGCs also express Cdh6 protein (Figures 3N–3Q). However, not all Cdh6 immunoreactive cells were Cdh3-RGCs (Figures 3P and 3Q), suggesting that Cdh6-RGCs represent a broader population of RGCs. Consistent with this idea, we obtained brains from Cdh6-GFP transgenic mice in which GFP is localized to axon terminals by Gap43-EGFP fusion (Inoue et al., 2009). Cdh6-RGCs heavily target the vLGN, IGL, OPN, and mdPPN, just like Cdh3-RGCs. However, Cdh6-RGCs also projected to the medial terminal nucleus (MTN) and the SC and the MTN itself expressed Cdh6 mRNA (Figure S3). Thus, Cdh3-RGCs selectively innervate Cdh6 expressing retinorecipient targets and Cdh6-RGCs project to those same targets, as well as to additional Cdh6-expressing targets.
Cadherin-6 Promotes Axon-Target Recognition in a Non-Image-Forming Visual Circuit

The most widely held view of cadherin-mediated cell-cell interactions is a homophilic model whereby cells expressing specific cadherin family members preferentially bind to cells expressing the same cadherin or combination of cadherins (Takeichi, 2007). Thus, we hypothesized that Cdh6 is involved in matching the axons of Cdh3/6-RGCs to Cdh6-expressing targets. To address this, we mated Cdh3-GFP transgenic mice to Cdh6 mutant mice (Dahl et al., 2002) to generate Cdh3-GFP::Cdh6+/− and Cdh3-GFP::Cdh6−/− mice. In early postnatal Cdh3-GFP::Cdh6+/− mice (n = 6 mice age P0/1; n = 6 mice age P5/6) and Cdh3-GFP::Cdh6−/− mice (n = 5 mice age P0/1 and n = 3 mice age P6), the axons of Cdh3-RGCs target the rostral pretectum normally and form dense oval terminal zones corresponding to the OPN (Figures 4A and 4B). Similarly, the axons of Cdh3-RGCs terminate in two dense foci in the caudal pretectum of early postnatal Cdh3-GFP::Cdh6+/− mice and Cdh6−/− mice (Figures 4D and 4E), a pattern typical of early postnatal projections to the mdPPN (Figure S1). In contrast, early postnatal Cdh3-GFP mice that lacked Cdh6 (Cdh3-GFP::Cdh6−/− mice) (n = 6 mice age P0/1; n = 5 mice age P6), had diminished axonal termination zones in the rostral pretectum (Figures 4C–4I). A similar lack of target recognition occurred in the caudal pretectum of Cdh3-GFP::Cdh6−/− mice: the axons of Cdh3-RGCs were abnormally dispersed along the margin of the optic tract and they failed to aggregate into foci typical of the early postnatal mdPPN (Figure 4F). We occasionally found Cdh3-RGC axons projecting to nonvisual midbrain areas; something we essentially never encountered in wild-type mice of the same ages (Figure 4J). In most cases, however, the Cdh3-RGC axons that failed to terminate in the OPN and mdPPN projected through and past these targets to form ectopic terminations in the more distal retinorecipient area, the SC (Figures 4G–4I). The ectopic SC terminations appeared in the deepest retinorecipient layer, the stratum opticum (asterisks in Figure 4I) or as terminal arbors in the overlying stratum griseum superficialis (Figure 4K). This stands in contrast to wild-type (Cdh6+/−) mice where Cdh3-RGC axons are rarely observed in the SC and when they are found there, they are generally confined to the caudomedial portion of the target (Figure 4G; asterisk). Together, these data indicate that when Cdh6 is deleted, the axons of Cdh3-RGCs fail to innervate their correct targets.

One interpretation of the defective target-innervation in Cdh3-GFP::Cdh6−/− mice is that it reflects a failure for Cdh3-RGCs to recognize their proper targets. We can rule out two alternative interpretations. First, the reduced OPN and mdPPN innervation did not result from reduced numbers of Cdh3-RGCs in the Cdh6 mutants because they were present in normal numbers compared to wild-type mice (Cdh6+/−: mean = 964 GFP RGCs; SEM = 57; n = 14 mice; versus Cdh6−/−: mean = 1020 GFP RGCs; SEM = 68; n = 4 mice) (p = 0.63, two-tailed student’s t test). Cadherins have been shown to play a role in sorting of motor neuron pools within the spinal cord (Price et al., 2002) which raises a second alternative interpretation of the mutant phenotype: that the axon targeting defects result from malformation of retinorecipient targets. To address this, we stained the brains of Cdh3-GFP::Cdh6−/− mice with antibodies to parvalbu-
synapses (Takeichi, 2007) supports that model. Alternatively, homophilic interactions among Cdh6 expressing RGCs may occur along the length of axons, en route to their targets. However, if the latter were the case, then we might expect to see defasciculation or axon growth deficiencies in Cdh6 mutants along the retinofugal pathway. We did not observe this; Cdh6 mutant axons arrived at their targets and indeed grew through and past them. They simply failed to terminate within those targets (e.g., Figures 4A–4I).

An alternative explanation is that the Cdh6 mutants phenotypes arise from heterophilic interactions among different cadherins. We did not examine Cdh6 binding specificity in this study,
but the expression of Cdh3 and Cdh6 in a single cohort of RGCs that innervate common targets (Figures 1–3), and the fact that Cdh2 is coexpressed with Cdh6 in those targets (Figures 1I and 1M), raises the possibility these cadherins generate target specificity by heterophilic interactions. The presence of multiple cadherins in the same neurons may also help explain why the Cdh6 null is not a fully penetrant phenotype: one cadherin may substitute in the others absence to reinforce proper axon-target connectivity. It is worth noting that age-dependent variability in phenotypes was also observed for kidney development in Cadherin-6 mutants (Mah et al., 2000).

Although not a fully penetrant phenotype, the absence of Cdh6 caused dramatic axon targeting defects in many cases, especially in early postnatal mice (Figures 4 and S4). The nature of those defects is informative toward understanding how cadherins impart specificity of connections: it was rare to observe mutant axons forming ectopic connections away from but in the vicinity of their normal targets. More often, the mutant axons traveled through their normal targets until they reached a different visual target, the SC. The fact that Cdh6 mutant axons grow through their targets but fail to stop and elaborate terminal arbors within them, supports the idea that removal of Cdh6 does not alter axon growth or guidance per se. Rather, Cdh6 appears necessary for axons to stop in the correct targets. The observation that misprojecting axons were able to invade the SC and form clustered terminations there (Figure 4I) also suggests that Cdh6 mutant axons are still capable of forming synapses. The location of those synapses is likely constrained by the guidance and activity dependent mechanisms that control afferent organization within that target, such as ephrins and spontaneous activity (Feldheim and O’Leary, 2010). Indeed, the retino-SC defects observed in Cdh6 mutants are reminiscent of the phenotypes observed in surgical “rewiring experiments” where RGC axons are forced into auditory nuclei. In those experiments, the misrouted RGC axons adopt terminal fields that are shaped by the local architecture and ephrin-based guidance systems they confront within the novel targets (Ellsworth et al., 2005).

Our data showing that an adhesion molecule is important for visual system wiring are consistent with previous reports showing that adhesion regulates RGC axon targeting at earlier points along the mammalian visual pathway (e.g., Williams et al., 2006) and with the abovementioned work in chicks and flies showing that N-Cadherin is important for lamina- and target-specificity (Inoue and Sanes, 1997; Lee et al., 2001; Prakash et al., 2005). Future studies that examine cadherin removal selectively in RGCs or in their targets, ought to shed further light on the mechanisms by which cadherins induce circuit specificity. In the meantime, our results provide evidence that cadherin mediated cell-cell adhesion is important for the establishment of functionally precise neural circuits in the mammalian visual system, by ensuring the appropriate sets of neurons in the eye connect to the appropriate sets of target nuclei in the brain.

**EXPERIMENTAL PROCEDURES**

**Animals**

Cadherin-3 BAC-EGFP (Cdh3-GFP) mice were obtained from MMIRC. Cdh6-GFP mice were generated as described in Inoue et al., (2009). Cdh6 knockout mice were made as described in Dahlgren et al. (2002) and obtained from stocks at Jackson laboratories. All procedures were carried out according to protocols approved by animal care and use committees at UCSD, UCSC, Stanford, and NYU.

**Immunostaining**

Immunostaining for GFP was carried out as described in Huberman et al. (2008) using rabbit anti-GFP (Invitrogen; 1:1000), rabbit anti-melanopsin (ATS; 1:2000), goat anti-CHAT (Chemicon; 1:100), rabbit anti-Cdh6 (Dr. Gregory Dressler University of Michigan; 1:500) or mouse anti-parvalbumin (Chemicon; 1:2000) and Alexa-488, -594, or -647 secondary antibodies.

**Labeling of Retinal Ganglion Cell Axons**

Methods identical to those described in Huberman et al. (2008) were used. Briefly, a 3 µl volume of 0.5% cholera-toxin beta (diluted in sterile saline) conjugated to Alexa-594 (Invitrogen) was injected into the right and left vitreous of Cdh3-GFP mice using a Hamilton syringe with 33 gauge needle. Twenty-four hours later, the mice were sacrificed, their brains removed and fixed for 24 hr in 4% PFA, then cryoprotected in 30% sucrose and sectioned at 35 µm in either the coronal or sagittal plane.

**In Situ Hybridization**

Complementary DNAs for Cdh1 (nucleotides 1624–2193 of the mouse mRNA, NM_009864), Cdh2 (nucleotides 2253–2800, NM007664), Cdh3 (nucleotides 836–1356, NM_007665), Cdh4 (nucleotides 530–1273, NM_009867), Cdh5 (nucleotides 722–1293, NM_009868), Cdh6 (nucleotides 202–1229 NM_007666), Cdh7 (nucleotides 1029–1517, NM_172653), and Cdh8 (nucleotides 214–1481, NM_007667) were used to make antisense and sense digoxigenin-tailed RNA probes. In situ hybridization as previously described (Feldheim et al., 1998); protease K treatments were 1 mg/ml for 1 min.

**Targeted Intracellular Filling of Ganglion Cells**

Cdh3-RGCs were targeted and injected with Neurobiotin in live retinal explants then fixed and reacted with streptavidin-Cy3 (see Bölgyi et al., 2009 for details).

**Retinal Ganglion Cell Quantification**

Cdh3-RGCs were counted from montaged high-resolution photomicrographs and then confirmed in the actual tissue specimen by comparison of the photo montages with the direct view of the retina on a Zeiss M1 microscope at 10–20 x magnification. Care was taken to only evaluate retinas where the entire whole mount was obtained by dissection. Student’s t tests were used for statistical comparisons of RGC numbers between wild-type and mutant retinae.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.neuron.2011.07.006.

**ACKNOWLEDGMENTS**

We thank Dr. Gregory Dressler for the cadherin-6 antibody and Tom Clandinin and Maureen Estevez for their helpful suggestions. This work was supported by NIH R01 EY014689 (D.A.F.), NIH R01 EY07360 (S.B.), NIH EY17832 to (B.V.), NIH R21 EY018320 and NIH R01 EY11310 (B.A.B.), and NIH R01 EY12793 (D.M.B.) and the E. Matilda Ziegler Foundation for the Blind (A.D.H.).

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Figure 2.S1: Cdh3-RGCs preferentially innervate Cdh6 expressing retinorecipient targets in the developing brain
(S1) Cdh3-RGCs preferentially innervate Cdh6 expressing retinorecipient targets in the developing brain.

(A-C) Axons from (A) all RGC axons (both eyes were injected with CTb-594), (B) Cdh3-RGCs and (C) their merge, in the visual thalamus of a P2 mouse. Cdh-3 RGC terminals are present in the ventral lateral geniculate nucleus (vLGN) and intergeniculate leaflet (IGL) but are scarce in the dorsal lateral geniculate nucleus (dLGN), with only fibers of passage readily apparent. dLGN=dorsal lateral geniculate nucleus; IGL=intergeniculate leaflet; vLGN=ventral lateral geniculate nucleus. (D-E) Axons from (D) all RGC axons and (E) Cdh3-RGCs in the P1 rostral pretectum. The optic tracts (OT) and two olivary pretectal nuclei (OPN) (arrows) are densely innervated by Cdh3-RGCs. (F,G) Axons from (F) all RGC axons and (G) Cdh3-RGCs in the P1 caudal pretectum. The two arrows indicate the medial division of the posterior pretectal nucleus (mdPPN), which is innervated by Cdh3-RGCs. Note there are many other retinorecipient pretectal nuclei (red in panel F) nearby, that Cdh3-RGCs do not project to. (D-G) Scale=200 μm. (H-J) High spatial correspondence between the pattern of: (H) CTb-594 labeled RGC axons, (I) Cdh3-GFP axons and, (J) Cdh6 mRNA in the OPN of a P1 mouse. (H-J) Scale=150 μm.
Figure 2.S2: Cadherin-3 is not expressed in retinorecipient targets and Cdh3-GFP BAC transgenic recapitulates endogenous Cdh3 expression
(S2) Cadherin-3 is not expressed in retinorecipient targets and Cdh3-GFP BAC transgenic recapitulates endogenous Cdh3 expression. (A,B) Cdh3 mRNA is absent from the rostral (A), and caudal (B) pretectum. This is not a failure to detect Cdh3 mRNA that was actually present because (C, D) show that Cdh3 mRNA is present just dorsal to the cerebral aqueduct of the same wildtype P1 mouse brain. (C) Low magnification image of the superior colliculus (SC) and the cerebral aqueduct (framed region) with Cdh3 mRNA expression in cells dorsal to the aqueduct. (D) High magnification view of framed region in panel (C). (E) Cdh3-GFP expressing cells dorsal to the aqueduct. Note that Cdh3-GFP expression matches Cdh3 mRNA expression (C,D), except that GFP labels entire cells because it is cytoplasmic. Scale=200μm (A,B). Scale=200μm (C). Scale=75μm (D,E).
Figure 2.S3: Cdh6-GFP RGCs project to the same non-image-forming visual targets as Cdh3-GFP RGCs, as well as the medial terminal nucleus and superior colliculus.
(S3) Cdh6-GFP RGCs project to the same non-image-forming visual targets as Cdh3-GFP RGCs, as well as to the medial terminal nucleus and superior colliculus.

(A-F) Axons of Cdh6-GFP axons in the (A) visual thalamus, (B, C) rostral prepectum OPN, (D) caudal prepectum mdPPN (arrows), (E) medial terminal nucleus (MTN) and (G) upper stratum griseum superficialis of the SC. Panel (F) shows Cdh6 mRNA in the MTN of a P4 mouse. The terminations of Cdh6-GFP RGCs are remarkably similar to terminations from Cdh3-GFP RGCs in the thalamus, rostral and caudal prepectum (see S2 and main Figures 1 and 2): they terminate densely in the vLGN, IGL, OPN (A-C) and mdPPN (arrows in panel D).

Asterisks in panels A indicate Cdh6-GFP axon terminals in brain regions that are not labeled in Cdh3-GFP mice. Along with Cdh6-GFP input to the MTN (E) and SC (G), these data indicate that Cdh6-GFP labels the same cohort of RGCs that express GFP in Cdh3-GFP mice as well as some additional RGCs. This is consistent with the Cdh6 antibody labeling of Cdh3-GFP retinas (main Figure 3) and with a recent report indicating that Cdh6-Cre RGCs include On-Off DSGCs (Kay et al., 2011), because previous work (Huberman et al., 2009; Rivlin-Etzion et al., 2011) has shown that mouse On-Off DSGCs project to the upper stratum griseum superficialis (panel G; uSGS) and the lateral margin of the dLGN (asterisk in panel A). On-Off DSGCs may also innervate MTN (Kay et al., 2011). Hipp=hippocampus. Note: This Cdh6 reporter mouse expresses GFP as a fusion to growth associated protein 43 (Gap43). Thus, GFP is localized to axon terminals and not to cell bodies or dendrites. GFP signal therefore cannot be used to gauge the locations of Cdh6 expressing neurons in retinorecipient targets. Scale=100μm for all panels except (B) where Scale=500μm.
Figure 2.S4: Variation in Cdh3-RGC axon mistargeting in late postnatal Cdh6<sup>−/−</sup> mice

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(D–E) The more severe phenotypes are associated with an increased number of ectopic terminations in the caudal pretectum. For example, panel D shows normal input to the OPN, and two small caudal pretectal foci (mdPPN) whereas panels E and F show progressively less input to the OPN, which is associated with increased number of ectopic terminations in the vicinity of the mdPPN. Scale=200μm.
(S4) Variation in Cdh3-RGC axon mistargeting in late postnatal Cdh6-/- mice.

Axonal terminations of Cdh3-RGCs in the OPN of six different Cdh3-GFP::Cdh6-/- mice, all age P20. (A-C) coronal plane of section showing rostral pretectum. (D-F) Sagittal plane of section showing rostral and caudal pretectum. Panels A and D show mutants with apparently normal targeting (compare to wildtype in Figure 2). Panels B, E show moderately defective target innervation patterns whereas panels C and F show severely defective projections, characterized by sparse Cdh3-RGC input to OPN and fragmented input to caudal pretectum (compare to main Figures 2 and 4). (D-E) The more severe phenotypes are associated with an increased number of ectopic terminations in the caudal pretectum. For example, panel D shows normal input to the OPN, and two small caudal pretectal foci (mdPPN) whereas panels E and F show progressively less input to the OPN, which is associated with increased number of ectopic terminations in the vicinity of the mdPPN. Scale=200um.
Supplemental Data References


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CHAPTER 3: Contactin-4 mediates axon-target specificity and functional development of the accessory optic system


Contactin-4 Mediates Axon-Target Specificity and Functional Development of the Accessory Optic System

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SUMMARY

The mammalian eye-to-brain pathway includes more than 20 parallel circuits, each consisting of precise long-range connections between specific sets of retinal ganglion cells (RGCs) and target structures in the brain. The mechanisms that drive assembly of these parallel connections and the functional implications of their specificity remain unresolved. Here we show that in the absence of contactin 4 (CNTN4) or one of its binding partners, amyloid precursor protein (APP), a subset of direction-selective RGCs fail to target the nucleus of the optic tract (NOT)—the accessory optic system (AOS) target controlling horizontal image stabilization. Conversely, ectopic expression of CNTN4 biases RGCs to arborize in the NOT, and that process also requires APP. Our data reveal critical and novel roles for CNTN4/APP in promoting target-specific axon arborization, and they highlight the importance of this process for functional development of a behaviorally relevant parallel visual pathway.

INTRODUCTION

Perception, cognition, and behavior all arise from highly precise patterns of connectivity between functionally specialized sets of neurons. Neural circuit precision emerges during development through a series of steps that collectively encompass a broad range of spatial scales. For example, axonal growth cones must navigate long distances, often many millimeters, to link distantly located structures. At the other extreme, growth cones have to select where to form synapses on the dendrites of target neurons with sub-micron specificity (Sanes and Yamagata, 2009; Lichtman and Denk, 2011). The ultimate goal of developmental neurobiology is to understand how specificity of circuit connections is established at all spatial scales and to determine how discrete alterations in specificity impact circuit function and behavior.

The cellular and molecular mechanisms underlying many steps of mammalian brain circuit assembly have been explored in detail. Prominent examples include growth cone navigation through intermediate choice points (Dickson, 2002), topographic mapping (Luo and Flanagan, 2007; Cang and Feldheim, 2013), and laminar specificity (Huberman et al., 2010; Robles and Baier, 2012; Baier, 2013). Among the lesser understood steps involved in circuit assembly, however, is axon-target matching. As growth cones navigate through the brain, they encounter many target nuclei; they must recognize which of those targets to innervate and which to avoid. While data on axon-target matching in the mammalian brain are starting to emerge (e.g., Osterhout et al., 2011, 2014; Schmid et al., 2014), our understanding of this crucial wiring step remains limited when compared with the other aspects of circuit wiring.

The vertebrate eye-to-brain pathway is a longstanding model for addressing how CNS axons achieve connection specificity. The diversity of retinal ganglion cell (RGC) subtypes and their associated patterns of connectivity with central visual system targets (Robles et al., 2014; Dhande and Huberman, 2014) provide an ideal model to dissect the mechanisms of axon-target matching. Mammalian RGCs include ~20 different subtypes, each responding to a specific feature in the visual world and connecting to a stereotyped set of retinorecipient targets (Morin and Studholme, 2014). The distinct parallel pathways created by precise RGC axon-target matching are what enable different features in the visual world to drive appropriate visual perception and behaviors (e.g., Güler et al., 2008; Sweeney et al., 2014), offering a unique opportunity to understand how axon-target matching relates to sensory circuit function and output.

The anatomical and functional specificity of a particular eye-to-brain circuit, the accessory optic system (AOS), makes it especially attractive for exploring axon-target matching in a behaviorally relevant context. The AOS evolved to control image stabilization and offset the visual “slip” that occurs when the head or eyes move at velocities too slow for the vestibular system to respond directly (reviewed in Simpson, 1984; Masseck and Hoffmann, 2009). The mammalian AOS consists of three target nuclei: the nucleus of the optic tract/dorsal terminal nucleus complex (NOT/DTN), the dorsal medial terminal nucleus ...
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**Figure 1. Contactin-4 Is Selectively Expressed by Axons of AOS-Projecting RGCs**

(A–C) Mouse visual pathway. Blue: AOS-projecting RGCs and axons. Magenta: retinorecipient targets. (A) Sagittal view, visual pathway, sf-AOT, superior fasciculus of the accessory optic tract; if-AOT, inferior fasciculus of the accessory optic tract; DTN, dorsal terminal nucleus; LGN, lateral geniculate nucleus. (B and C) Coronal views of AOS targets. (B) The NOT and (C) the medial terminal nucleus, dorsal (MTNd), and ventral (MTNv) divisions.

(D–I) Contactin-4 (CNTN4) protein expression in AOS targets, the NOT and MTN. (D) CTβ-594 (magenta) labeling of all RGC axons. (E) CNTN4 protein (green) is selectively expressed in the NOT. (F) Merge of (D) and (E). Asterisks represent retinorecipient targets that do not express CNTN4. Scale represents 250 μm. (G) CTβ-594 labeling all RGC axons. (H) CNTN4 protein expression in the medial terminal nucleus (MTN). Asterisk represents CNTN4 expression outside of the visual system. (I) Merge of (G) and (H). Scale represents 250 μm.

(J–L) CNTN4 protein in axons in optic tract (OT). (J) CTβ-594/CNTN4 labeling. Scale in (J) represents 200 μm. (K) High-magnification view framed region from (J). L) Merged high-magnification view of CTβ-594 and CNTN4. Scale in (K) and (L) represents 100 μm. Arrows in (K) and (L) represent CNTN4+ axonal profiles. Asterisk represents CNTN4 expression outside of the visual system.
(MTNd), and the ventral medial terminal nucleus (MTNv), which is also sometimes referred to as the lateral terminal nucleus (LTN) (Figures 1A–1C) (Simpson, 1984; Pak et al., 1987; Yonehara et al., 2009; Dhande et al., 2013). These nuclei receive input from a functionally specialized collection of RGC subtypes: three subtypes of On-direction selective RGCs (On-DSGCs) and one subtype of On-Off DSGCs, all of which are tuned to slow speeds (Yonehara et al., 2009; Dhande et al., 2013). The axons that project to the NOT target this region by terminal arbors or by collateral branching of an axon en route to the more distal DTN and/or superior colliculus (SC), whereas RGC inputs to the MTN arise solely from terminal arbors that arrive via the accessory optic tracts (Figures 1A–1C) (Yonehara et al., 2009; Dhande et al., 2013). While progress has been made in identifying the RGC subtypes and targets that comprise the mammalian AOS (Yonehara et al., 2009; Kay et al., 2011; Dhande et al., 2013), the cellular mechanisms controlling development of this crucial visual circuit have only recently been investigated (Osterhout et al., 2014), and the molecular mechanisms are still completely unknown.

Here we explored the molecular mechanisms of axon-target matching in the assembly of the mammalian AOS. Using genetic labeling of AOS-projecting RGCs, knockout mouse analyses, and single-cell overexpression experiments, we show that the IgG superfamily member contactin 4 (CNTN4/BIG-2) is necessary for AOS-projecting RGC axons to innervate the AOS target required for horizontal image stabilization (the NOT) and is sufficient to bias RGCs to selectively arborize in that target—a process that is contingent on expression of amyloid precursor protein (APP). Our findings reveal that axon-target matching in the mammalian brain involves target-specific axonal arborization that is ultimately crucial for the function of brain circuits linked to specific behaviors.

RESULTS

Contactin-4 Is Expressed by RGCs that Target AOS Nuclei

To explore the molecular signals controlling development of parallel eye-to-brain circuits, we screened the expression patterns of IgG superfamily proteins in retinorecipient targets and identified Contactin-4 (CNTN4) as a candidate. CNTN4 belongs to a small family of axon-associated cell adhesion molecules within the IgG superfamily that has six Ig domains and four fibroectin type III domains and is glycosphatidylinositol (GPI)-anchored to the plasma membrane (Yoshihara et al., 1995; Kaneko-Goto et al., 2008; reviewed in Shimoda and Watanabe, 2009). Labeling of retinorecipient targets by intravitreal injections of cholera toxin beta (CTβ)-594 followed by staining of tissue sections with an antibody specific for CNTN4 (Kaneko-Goto et al., 2008; Figures S1E and S1H) revealed that it was selectively expressed in AOS targets: it was present at high levels in the developing NOT (Figures 1D–1F) and at lower levels in the MTNd and MTNv (Figures 1G–1I). In contrast, there was little or no CNTN4 protein expression in other retinorecipient nuclei (Figures S1A–S1G), even those situated adjacent to NOT (Figure 1F, asterisks).

CNTN4 protein expression was absent from RGC somas, but was clearly expressed by a small subset of RGC axons within the optic tract (Figures 1J–1L), which is consistent with previous findings that CNTN4 expression is axonally localized (Yoshihara et al., 1995). To determine whether CNTN4 expression in AOS targets arises from RGC axons, we removed one eye from post-natal day 0 (P0) mice and allowed a period of 7 days for RGC axons from that eye to degenerate. We then labeled the RGC axons from the intact eye with CTβ-594 and compared the patterns of CNTN4 expression in AOS targets on the two sides of the brain at P8. CNTN4 protein was present in AOS targets contralateral to the intact eye (Figures 1M, 1N, 1R, 1S) but was absent in AOS targets contralateral to the enucleated eye (Figures 1P, 1Q, 1U, 1V). Thus, we conclude that CNTN4 expression in AOS targets arises from RGC axons.

Developmental Expression of CNTN4 Protein in the NOT

Next we analyzed the timing of CNTN4 expression in AOS targets during the developmental time frame when RGC axons arrive to these targets. CNTN4 protein expression was weak in the NOT from P1–P5 (Figures 2A and 2B) but increased by P8 (Figure 2C), the stage when most AOS-projecting RGC axons innervate this target (Osterhout et al., 2014). After P8, CNTN4 protein expression in the NOT diminished (Figure 2D). We also analyzed CNTN4 expression in the NOT of P8 Hoxd10-GFP transgenic mice in which all four subtypes of AOS-projecting RGCs (three On-DSGC subtypes and one On-Off DSGC subtype) selectively express GFP (Dhande et al., 2013). The Hoxd10-GFP+ axonal profiles overlapped with CNTN4 protein in the NOT (Figures 2E–2G). In addition, the specificity of CNTN4 expression in the entire visual pathway matched the projection pattern of Hoxd10-GFP RGC axons (Figure S1). Taken together, these data indicate that CNTN4 is expressed by the axons of AOS-projecting RGCs during the developmental phase when they grow into their targets in the brain.

Reduced Innervation of the NOT by AOS-DSGC Axons in CNTN4 Mutant Mice

Does CNTN4 play a functional role in generating axonal connectivity between AOS-projecting RGCs and their targets? To test this, we crossed CNTN4 null mutant mice (CNTN4+/−; Kaneko-Goto et al., 2008) to Hoxd10-GFP reporter mice and analyzed the projection patterns of the GFP-expressing axons at P8 and P20. In WT (CNTN4+/+), Hoxd10-GFP mice the GFP+ axons densely filled the entire NOT at both P8 and P20 (Figures 3A and 3D). In contrast, there was a reduction in the density of NOT-projecting Hoxd10-GFP RGC axons in CNTN4−/−, Hoxd10-GFP+/− mice.
mice (Figures 3B and 3E) and in CNTN4−/−, Hoxd10-GFP mice (Figures 3C and 3F). At P8 and P20, a small number of Hoxd10-GFP RGC axons still terminated along the lateral edge of the NOT (e.g., Figure 3F), but there was a clear and consistent defect in overall innervation density within this target. This defect was confirmed by comparison of the percentage of NOT area occupied by Hoxd10-GFP RGC axons in CNTN4 mutant mice versus their WT littermates at both P8 and P20 (Figure 3G; p < 0.05, n = 4–6 mice per genotype, per age; see Experimental Procedures). In contrast, there were no observable changes in the pattern of projections from Hoxd10-GFP RGC axons to the MTNd or MTNv (Figures S2A–S2C). In addition, bulk whole-eye labeling of RGC axons showed that the timeframe of NOT innervation was unchanged in CNTN4 mutants (Figures S2D–S2G). Dual-color whole-eye labeling also showed that the pattern of binocular RGC targeting to other retinorecipient targets also appeared normal (Figures S2H–S2K).

One possible explanation for the axonal projection phenotype we observed in the NOT is that there was a reduction in the number of Hoxd10-GFP RGCs present in CNTN4 mutants. However, when we quantified the total number of Hoxd10-GFP RGCs in WT CNTN4+/+ mice and in CNTN4−/− mice at P8, we found no significant differences between these groups (Figure 3H). Additionally, the dendritic stratification patterns of Hoxd10-GFP RGCs appeared normal (Figures S2L–S2S). We also studied the electrophysiological properties of Hoxd10-RGCs in retinal whole mounts from WT and CNTN4 mutant mice (Figure S3). In both WT and CNTN4 mutant retinas, Hoxd10-GFP RGCs included the three expected On-DSCGs subtypes and one On-Off DSCGs subtype (Figures S3A and S3B), all of which were tuned to the correct axes of motion and displayed no significant differences in magnitude of direction tuning compared with control Hoxd10-GFP RGCs (Figures S3E and S3F). Together, our data indicate that loss in CNTN4 expression results in perturbed projections to the NOT by AOS-projecting RGCs and that the altered patterns of NOT innervation are not the consequence of changes in Hoxd10-GFP RGC number, subtype identity, or retinal wiring.

Interestingly, although Hoxd10-GFP RGCs comprise the majority of AOS-projecting RGCs (Dhande et al., 2013), whole eye labeling showed that the total volume of projections to this target was normal in CNTN4−/− mice (Figures 3I–3K; n = 3 mice per genotype), suggesting non-AOS RGCs fill in the target zone. Indeed, when we explored the axon targeting patterns of another category of RGCs, the On-Off DSCGs labeled in DRD4-GFP transgenic mice (Huberman et al., 2009), we found they had abnormal NOT projections in CNTN4 mutant mice (Figures 3L–3R). In mature WT DRD4-GFP mice, the GFP+ RGCs project to the NOT only transiently during development, and by P20, they have retracted from the NOT (Figures 3L, 3O, and 3R) (Huberman et al., 2009; Kay et al., 2011; Osterhout et al., 2014). By exploring multiple ages of CNTN4−/−, DRD4-GFP mice, we discovered that CNTN4 mutations cause an abnormal maintenance of DRD4-GFP RGC projections to the NOT (Figures 3M, 3N, 3P, and 3Q). Targeting of DRD4-GFP RGC axons to the dLGN and SC in contrast appeared normal (Figures S4A–S4D), indicating that the impact of CNTN4 mutations on these RGCs was AOS target specific.

To determine whether other RGCs subtypes also alter their axon projection patterns in CNTN4 mutant mice, we crossed CNTN4 mutants to Cdh3-GFP mice. These mice selectively express GFP in a subset of intrinsically photosensitive RGCs (ipRGCs) that project to non-image-forming targets important for pupil reflex and circadian-related behaviors (Osterhout et al., 2011, 2014). The projection patterns of Cdh3-GFP RGC axons were unaltered in CNTN4 mutants (Figures S4E–S4L), and they avoided the NOT (Figures S4K and S4L), just as they typically would in WT mice (Osterhout et al., 2011, 2014). Together, the analyses of Hoxd10−/−, DRD4−/−, and Cdh3-GFP mice carrying CNTN4 mutations indicate that the developmental influence of CNTN4 on RGC axon-target matching is limited to the axons that interface with AOS targets during development.

Axonal Arborization as a Key Step in CNTN4-Mediated Axon-Target Matching

To gain a better understanding of the mechanisms by which CNTN4 mediates targeting of RGC axons to the NOT, we tested whether the phenotypes present in CNTN4 mutants reflected a decrease in the number of axons targeting the NOT or decreased arbor complexity of correctly targeted axons. Either theory could result in an apparent reduction of Hoxd10−/− GFP axons in the NOT. We used sparse in vivo RGC electroporation (Dhande et al., 2013), a method that yields a sparse population of electroporated RGCs. We stained these cells with GFP using electroporation followed by immunohistochemistry and then quantified the number of GFP-labeled RGC axons innervating the NOT (Figures 3I–3K). Despite the sparse population of electroporated RGCs, the total volume of axons labeling the NOT was normal in CNTN4−/− mice (Figures 3I–3K; n = 3 mice per genotype), suggesting that the decrease in NOT area innervation in CNTN4−/− mice (Figures 3C and 3F) was not due to decreased axon number but rather to decreased axon arbor complexity.
to ectopically express tdTomato in individual RGCs of WT and CNTN4 mutant mice at P0 and then examined their axons in the brain 1 week later, on P8 (Figures 4A and 4A’). Figures 4B and 4C show an example of an electroporated RGC expressing tdTomato throughout its cell body, dendritic arbor, and axon. By sparse-electroporating large numbers of WT mice, we succeeded in labeling four individual NOT-projecting RGC axons in separate animals. Labeling of individual NOT-projecting RGC axons in CNTN4+/− mice proved even more challenging. We did, however, successfully label two individual NOT-innervating RGC axons in CNTN4+/− mice (e.g., Figures 4F and 4G). Reconstruction and quantification of the morphological features of these axons (branch number, arbor area, etc.) revealed that WT NOT-targeted RGC axons were significantly more complex than CNTN4 mutant axons (compare Figure 4E and 4G; quantified in Figure 4H). The surface area and volume of CNTN4−/− arbors were also reduced compared with WT (Figures 4I and 4J).

Notably, the fraction of electroporated RGCs that projected to the NOT was also greatly reduced in CNTN4 as compared with WT mice (Figure 4K). These data suggest that the phenotype of reduced Hoxd10-GFP RGC input to the NOT (Figures 3A–3F) likely arises from two sources: (1) a reduction in the number of AOS-projecting RGCs targeting the NOT and (2) reduced branching and overall complexity of the arbors that do manage to innervate this target.

**Ectopic Expression of CNTN4 Biases RGCs to Branch in an AOS Target**

To further explore the role of CNTN4 in RGC axon-target matching, we again utilized sparse in vivo RGC electroporation to ectopically express either control, tdTomato plasmid or a plasmid encoding full-length CNTN4 in RGCs that normally bypass the NOT. We accomplished this by electroporating one or the other plasmid into the retina of Hoxd10-GFP mice at P0 and examined the targeting of non-Hoxd10-GFP RGC axons in the brain a week later, on P8. Of the 58 control/tdTomato+ RGC axons we examined, 8 of them (~14%) innervated the NOT; the remaining 50/58 tdTomato+ axons traveled through or over the NOT to arborize in more distal retinorecipient targets such as the SC (Figures 5A–5C and 5H). In contrast, ectopic expression of CNTN4 in individual RGC axons strongly biased them to arborize in the NOT; 60% of CNTN4-electroporated RGCs (12/20) targeted and electroporated axonal arbors in the NOT (Figures 5D and 5E)—a nearly 5-fold increase over what was observed for control RGCs electroporated with tdTomato (Figure 5H; p = 0.0002). We note that 10/12 of the CNTN4+ RGC axons that elaborated arbors in the NOT also projected to the SC. Thus, CNTN4 expression in individual RGC axons biases them to form axonal arbors in the NOT but does not appear to otherwise alter their growth or patterning of retinorecipient targeting.

The results of our expression analyses described above indicate that normally CNTN4 is expressed by a subset of AOS-projecting RGC axons but not by cells in retinorecipient targets. In theory, however, CNTN4 expressed by other RGC axons could bias RGCs to elaborate arbors in the NOT. To test this, we electroporated CNTN4 into individual RGCs in CNTN4+/− mice and examined their resulting pattern of axon targeting in the brain. Remarkably, even in the CNTN4+/− background, ectopic expression of CNTN4 in an individual RGC axon strongly biased that axon to arborize in the NOT. Approximately 57% (12/21 axons) of the CNTN4-electroporated RGCs arborized in the NOT in CNTN4+/− mutants (Figures 5F–5H), which was not significantly different from the impact of ectopically expressing CNTN4 in mice of WT background (Figure 5I).

We also addressed the possibility that CNTN4 non-specifically promotes axon arborization independent of target region by comparing the frequency of innervation within other major visual targets by axons electroporated with tdTomato or CNTN4 plasmid. The frequency of arborization in the ventral and dorsal lateral terminal nuclei (vLGN and dLGN, respectively) or the SC was indistinguishable between control and CNTN4-electroporated groups (Figure 5I). Thus, the expression of CNTN4 in an individual RGC axon biases that axon to target and establish arbors specifically in the NOT.

**APP Is Necessary for CNTN4-Mediated AOS Targeting**

What are the possible binding partners required for CNTN4’s influence on axon targeting to the NOT? Previously, the Flanagan lab used biochemical and in vitro growth cone assays to discover the binding partners of APP and found that CNTN4 is one of the major binding partners in chick neurons and that CNTN4 can impact RGC growth cone guidance (Osterfield et al., 2008). Intrigued by those findings, we sought to determine whether CNTN4 and APP interact in mice and contribute to targeting of RGCs to the AOS. We first co-immunoprecipitated APP from P8 whole-brain lysate and immunoblotted for CNTN4 protein. We were able to pull down CNTN4 protein with APP using WT tissue, but not CNTN4 mutant tissue (Figures 6A and 6B). While these experiments do not rule out other binding partners for APP or CNTN4, they establish a direct relationship between the two. We next analyzed the developing visual pathway for APP protein by immunohistochemistry and found that it is expressed by the vast majority of cells within the developing ganglion cell layer of the retina, including Hoxd10-RGCs (Figures 6C and S5I–S5K). APP protein is also expressed within most retinorecipient targets, including the NOT, where it overlaps with the expression of CNTN4 (Figures 6D–6F). Eye removal abolished APP staining in the NOT (Figure 6G), indicating that, like CNTN4, APP is expressed by RGC axons that target the NOT and not by cells within the NOT.

To examine the role of APP in RGC axon-target matching in the AOS, we generated APP−/−, Hoxd10-GFP mice and analyzed the projection patterns of the GFP+ RGCs in the brain. The density and extent of Hoxd10- RGC axon targeting in the NOT was significantly reduced in both APP−/−, Hoxd10-GFP and APP+/−, Hoxd10-GFP mice compared with their WT littermate controls (Figures 6H–6J and 6L; n = 4–5 mice per genotype). Indeed, the fraction of the termination zone occupied by Hoxd10-GFP RGC axons was qualitatively and quantitatively similar between CNTN4 and APP heterozygous and homozygous null mice (Figure 6L). Similar to CNTN4 mutants, Hoxd10-GFP RGC inputs to the MTN and whole-eye innervation and binocular segregation of RGC projections to the visual thalamus and SC appeared normal in APP mutant mice (Figures S5A–S5C and 5E–5G). Also, the number of Hoxd10-RGCs was comparable between APP mutant
Figure 3. Loss of CNTN4 Results in a Decrease of Innervation by Hoxd10-GFP RGC Axons to the NOT

(A–F) Hoxd10-GFP RGC axons in the NOT at P8 (A–C) and at P20 (D–F) in WT (A and D), CNTN4<sup>+</sup>/<sup>−</sup> (B and E), and CNTN4<sup>−/−</sup> mice (C and F). Scale represents 250 μm. D, dorsal; L, lateral.

(G) Fraction of the termination zone (in NOT) occupied by Hoxd10-GFP RGC axons in WT (black), CNTN4<sup>+</sup>/<sup>−</sup> (dark gray), and CNTN4<sup>−/−</sup> (light gray) mice (± SEM). *p < 0.05 (n = 4–6 mice per genotype, per age).

(H) Total number of Hoxd10-RGCs in the retinas of WT and CNTN4<sup>−/−</sup> mice at P8 (± SEM). n = 5–6 mice per genotype.

(I and J) RGC axons in NOT of WT (I) and CNTN4<sup>−/−</sup> mice (J) labeled by intraocular CT<sub>b-594</sub>. Scale represents 250 μm.

(K) Total volume of the NOT (mm<sup>3</sup> ± SEM) in WT (black) and CNTN4<sup>−/−</sup> mice (gray) (n = 3 mice per genotype).
Figure 3.4: CNTN4 is required for accurate targeting and efficient arborization in the NOT and WT retinas (Figure S5D). Thus, altering APP expression does not generally perturb RGC targeting or survival, but it does have a significant impact on axon-target matching to the AOS in a manner resembling CNTN4.

To address whether APP and CNTN4 mediate axon-target matching of AOS-projecting RGCs through convergent or independent molecular pathways, we generated double-mutant, transgenic CNTN4+/−/−, APP+/−/−, Hoxd10-GFP mice. Our prediction was that if CNTN4 and APP reside in independent pathways then removal of both of them would cause an additive reduction to NOT targeting beyond that observed in APP or CNTN4 single mutants alone. Instead, however, we observed a similar reduction in Hoxd10-GFP RGC innervation of the NOT in CNTN4+/−/−, APP+/−/− double-mutant mice as we observed in CNTN4-only or APP-only mutant mice (Figures 6K and 6L).

To further address the role of APP in CNTN4-mediated RGC targeting, next we used an in vivo genetic approach. We ectopically expressed CNTN4 in individual RGC axons by (L–Q) DRD4-GFP RGC axons in the NOT at P8 (L–N) and at P20 (O and P), in WT (L and O), CNTN4+/−/− (M and P), and CNTN4+/− mice (N and Q). Scale represents 250 μm.

(R) Fraction of the termination zone (in NOT) with DRD4-GFP RGC axons in WT (black), CNTN4+/− (dark gray), and CNTN4+/− (light gray) mutants (± SEM). *p < 0.05, **p < 0.01 (n = 5–6 mice per genotype per age).

Figure 4. CNTN4 Is Required for Accurate Targeting and Efficient Arborization in the NOT

(A and A') In vivo electroporation. (A) Plasmid DNA is injected into the eye on P0 and receives square waves pulses. (A') P8 brains are examined for labeled RGC axons.

(B) Example of pCMV-tdTomato electroporated RGC. (Arrowhead and inset) RGC axon expressing tdTomato.

(C) High magnification of the RGC shown in (B) the RGC soma, dendrites, and axon (arrowhead) expressing high levels of tdTomato. Scale represents 125 μm.

(D–G) Example of NOT-projecting tdTomato+ axons in WT (D and E) and CNTN4−/− mice (F and G); arrowhead represents parent axon. (E) WT, NOT-projecting RGC axon reconstruction from boxed region in (D). (G) CNTN4−/−, NOT-projecting axon reconstruction from boxed region in (F). Scale in (D) and (F) represents 250 μm. Scale in (E) and (G) represents 50 μm.

(H) Quantification of the average number (± SEM) of branches from branch order 1–8 for WT and CNTN4−/−, NOT-projecting axon (n = 4 axons/mice WT, n = 2 axons/mice CNTN4−/−).

(I) Quantification of the average arbor surface area for WT and CNTN4−/− mice (± SEM), p = 0.040.

(J) Quantification of the average arbor volume for WT and CNTN4−/− mice (± SEM), p = 0.062.

(K) The percentage of electroporated axons arborizing in the NOT after electroporation in WT (8/58) and CNTN4−/− (2/25) mice.
Figure 3. CNTN4 expression in RGC axons is sufficient to bias arborization in the NOT

(A–G) Electroporation of RGCs with control/pCMV-tdTomato plasmid (A–C) or CNTN4 plasmid (D–G). (A) Td-tomato+ axon (magenta) projecting through the NOT in a Hoxd10-GFP mouse (green). Scale represents 250 μm. (A’) High magnification of tdTomato+ RGC axons from the boxed region in (A). Scale represents 125 μm. (B) Schematic of typical outcome of td-Tomato expression (red) in RGCs of WT mice (WT denoted by blue color). (C) Axon terminal in the SC; this is the same axon as shown in (A) and (A’). (Inset) High magnification of boxed region in (C). Scale represents 250 μm. lSGS, lower stratum griseum superficiale; uSGS, upper stratum griseum superficiale. (D–E) Example RGC axon electroporated with pEF-CNTN4 plasmid in WT background. (D’) High magnification of axon in (D). (E) Schematic of typical outcome of electroporation of CNTN4 in RGCs in a WT background. (F and G) Example RGC axon electroporated with pEF-CNTN4 in a CNTN4−/− mouse. (F’) High magnification of axon in (F). (G) Schematic of typical outcome of electroporating CNTN4 into RGCs of CNTN4−/− mice. Scales in (D’) and (F’) represent 125 μm.

(H) Percentage of electroporated RGC axons arborizing in the NOT after electroporation of tdTomato or CNTN4. Statistical significance is determined by Fisher analysis, ***p < 0.001, ****p < 0.0001; the fraction of electroporated RGCs for each experiment is given at the bottom of the bars.

(I) Percentage of wild-type electroporated axons arborizing in other major retinorecipient targets after electroporation of tdTomato or CNTN4. Statistical significance is calculated using Fisher analysis.

Ectopic expression of tdTomato

Ectopic expression of CNTN4

Functional Defects in AOS Circuits and Their Behavioral Outputs in CNTN4 Mutant Mice

What are the functional consequences of altering Hoxd10-RGC input to the NOT? The NOT is responsible for generating horizontal compensatory eye movements in response to slow speed motion (Simpson, 1984). To determine whether there are defects in the activity of NOT neurons in CNTN4−/− mice, we presented dark-adapted head-fixed WT mice and CNTN4−/− mice with...
Figure 3.6: APP is a CNTN4 binding partner and is required for normal RGC targeting in the NOT horizontal or vertical stimuli optimal for driving slip-compensating eye movements (Figure 8A) (Dhande et al., 2013). Mice were exposed to 2 hr of stimulation with a sham stimulus (gray screen), a horizontal motion stimulus, or a vertical motion stimulus and then were perfused and their brains processed for c-Fos immunoreactivity, which is an indirect readout of neural activation (Omori et al., 2005; Yonehara et al., 2009). In WT mice, large numbers of intensely labeled c-Fos + cells were observed in the NOT after horizontal motion stimulus as compared with a sham stimulus (Figures 8B and 8C). However, in CNTN4 −/− mice shown an identical horizontal motion stimulus, the number of c-Fos + cells was significantly reduced, and the few c-Fos + cells that were activated appeared dimmer (Figures 8D and 8E). Notably, the number of c-Fos + cells in the NOT in response to vertical stimulation was similar to the number of c-Fos + cells elicited by sham stimulus (Figure 8E) and c-Fos activation in a different retinorecipient target, which responds to overall ambient luminance but not to motion, the suprachiasmatic nucleus (SCN), revealed no significant differences between WT or CNTN4 −/− mice using either sham or horizontal motion stimulus (Figures 8F–8I). These data indicate that loss of CNTN4 causes a significant defect in the activation of NOT neurons in response to stimuli that normally drive this retinofugal pathway.

What are the behavioral consequences of reduced afferent input to the NOT from AOS-RGCs in CNTN4 mutants? To address this, we measured optokinetic reflex (OKR) behavior where mice display compensatory head movements in response to bar-grating stimuli drifting slowly in either the horizontal or vertical axis (Figures 8J, 8J’, and 8K; Prusky et al., 2008; Wang et al., 2009; Tschetter et al., 2011). We quantified the percentage of 15-s horizontal or vertical motion trials tracked by P25-P30 WT and CNTN4 −/− mice (Figures 8J–8L). WT mice tracked 95% of the horizontal-stimulus trials and tracked 80% of the vertical-stimulus trials (Figures 8J and 8K; n = 5 mice), whereas CNTN4 −/− mice tracked only 50% of horizontal trials and 60% of vertical trials, both of which represent significant reductions in OKR behavior compared with controls (Figures 8K and 8L; n = 5 mice; p < 0.01). These differences are highly unlikely to be caused by defects in retinal wiring as the number and physiology of RGCs that drive this system was normal in CNTN4 mutants at equivalent ages (Figures S3 and S5G). These indicate that the reduction in Hoxd10-GFP RGC projections to the NOT and the diminished c-Fos activation of NOT neurons are both also associated with a defect in AOS circuit performance.

Figure 6. APP Is a CNTN4 Binding Partner and Is Required for Normal RGC Targeting of the NOT
(A) Schematic of proposed interaction between CNTN4 and APP (Osterfield et al., 2008).
(B) Co-immunoprecipitation: APP protein was immunoprecipitated from WT or from CNTN4 −/− brain tissue and was run on a western blot that was blotted for CNTN4 and APP antibodies.
The defects in vertical OKR tracking in CNTN4−/− mice may seem somewhat surprising given that Hoxd10-GFP RGC input to the MTN appears normal in these mice (Figures S2B and S2C); it is noteworthy that NOT neurons are known to project to and modulate activity of a subset of neurons in the MTN (Simpson, 1984). Indeed, in WT mice, there were a greater number of c-Fos+ cells in the MTN after horizontal full-field motion as compared with a sham stimulus, and this effect was lost in CNTN4−/− mice (Figures S6A and S6C–S6E). Importantly, there were no defects in the pupillary light reflex in CNTN4−/− mice, even when tested at several irradiance levels (Figure S6F). Together, these data indicate that loss of CNTN4 and the associated defects in axon-target matching to the NOT selectively perturb AOS circuit function and its behavior-generating capacity.

**DISCUSSION**

Here we report a molecular mechanism controlling a specific and functionally essential aspect of parallel pathway assembly: axon-target matching. We identified CNTN4 as a factor required for a subset of RGCs to connect to AOS brain targets for image stabilization. Then, through loss-of-function and gain-of-function experiments in WT, CNTN4, and APP mutant mice, we determined that (1) CNTN4 expressed by RGC axons is required for target-specific arborization of those RGCs in the NOT, (2) this process requires APP, and (3) functional and behavioral consequences result from disruption of axon-target specificity in this pathway.

**Axon-Target Matching by Target-Specific Arborization**

By exploring the effects of ectopically expressing CNTN4 in individual RGCs, we identified key aspects of the axon-target matching process in the developing optic pathway: target-specific axon arborization. In mice >90% of RGCs project to the most distal retinorecipient target, the SC, and the vast majority of projections to other retinorecipient targets represent locally arborized collateral branches of those same SC-projecting axons (Hofbauer and Dräger, 1985; Huberman et al., 2009; Dhande et al., 2011). When we electroporated RGCs with CNTN4, we found that the axons of those RGCs tended to establish branched arbors specifically in the NOT. We found that their axons still traveled to the SC where they formed arbors and that there was no increased propensity to establish arbors in other major retinorecipient targets—indicating a role in specificity as opposed to non-specific growth. Our results suggest several possible mechanisms by which CNTN4 exerts its effects on AOS wiring. CNTN4 may promote NOT targeting, which in turn initiates arbor formation, or CNTN4 may promote axon arborization in the NOT in a manner that reflects “targeting” as the final outcome. In the absence of in vivo time-lapse imaging, these two possibilities are difficult to separate. Nonetheless, given that CNTN4 mutations both reduced the frequency of NOT targeting and reduced arbor complexity, it is clear that these processes are linked. Taken with previous findings, these results indicate that axon-target matching reflects molecular programs that can bias axons to (1) locally steer toward groups of targets (e.g., Ringstedt et al., 2000), (2) remain within those targets...
Figure 3.8: Loss of CNTN4 perturbs function and behavioral output of AOS circuitry once they arrive (Sun et al., 2015 [in this issue of Neuron]; Osterhout et al., 2011), and (3) promote target-specific arborization (the present data). Recent work in Drosophila also underscores the extent to which axon-target matching is a multistep process that reflects a series of axon-navigation and arbor maturation events (Joo et al., 2013).

What factors control RGC targeting to the MTN? An accompanying paper describes a key role for semaphorin-plexin reverse signaling in the development of retino-MTN circuitry (Sun et al., 2015). It is possible that semaphorin-plexin signaling may override the requirement for CNTN4 in the MTN-projection pathway.

Figure 8. Loss of CNTN4 perturbs function and behavioral output of AOS circuitry

(A) Schematic of visual stimulation. Head-fixed mouse is placed in a chamber surrounded by video monitors on all four sides. (B–E) Analysis of c-Fos expression in NOT cells to assess the level of activity induced by a visual stimulus in WT (B and C) or CNTN4−/− mice (D). (Insets in B–D) High-magnification views of boxed regions. Scale represents 250 μm. (E) Number of c-Fos+ cells in the NOT per mm3 (± SEM). **p < 0.01 (n = 5–6 mice per group; age is P22–P23).

(F–I) c-Fos activation in the SCN of WT (F and G) or CNTN4−/− (H) mice. Scale represents 100 μm. (I) Number of c-Fos+ cells per mm3 of the SCN (± SEM); ns, no significant difference.

(J–L) Schematic of the OKR behavioral analysis; head movements in response to horizontal (J) or vertical drifting stimuli (J') measured (see text and Experimental Procedures). (K) The average percentage of trials tracked by CNTN4+/- or CNTN4−/− mice in response to horizontal or vertical motion (± SEM) (n = 5 mice per genotype). (L) Plot of percentage of trials tracked in vertical versus horizontal motion for each animal.

Also, it is notable that DSGC inputs to the MTN comprise a distinct axonal trajectory from the other AOS-projecting RGCs and may require unique molecular cues (e.g., Figure 1A; Pak et al., 1987; Yonehara et al., 2009; Dhande et al., 2013; Dhande and Huberman, 2014). It is also possible that CNTN4 is important for arborization in the MTN, but the compact, “tract-like” architecture of this target nucleus makes it challenging to detect abnormal arborization patterns within it. Nevertheless, we note that ectopic expression of CNTN4 in individual RGC axons did not bias RGC axons to terminate in the MTN, suggesting that other pre-target and within-target molecular signals govern
APP Is Required for CNTN4-Mediated RGC Axon Targeting

Our data reveal a novel role for APP in axon-target matching. Removal of APP caused an overall reduction in NOT innervation by Hoxd10-GFP RGCs. Removal of APP was also sufficient to occlude CNTN4-mediated biasing of RGC axon arborization in the NOT. Since we find that APP is expressed by most RGCs, it seems unlikely that APP directly imparts target specificity. Rather, because both APP and CNTN4 are expressed by RGC axons, we favor a model in which APP acts as co-receptor with CNTN4 to allow CNTN4 to impart its role in target specificity. At this time, the identity of the putative target-derived ligand is unknown, but there is a general precedent for this model; Kaltschmidt and co-workers recently showed that a closely related contactin, CNTN5, operates with a co-receptor to influence axon connectivity in the developing vertebrate spinal cord (Ashrafi et al., 2014). It is also interesting that both in our study and that of Ashrafi et al. (2014) axonal targeting defects were observed in heterozygous and homozygous CNTN mutant mice, which points to the importance of maintaining correct CNTN levels for CNS wiring.

The fact that whole-eye labeling of all RGCs in APP mutant mice did not reveal any overt changes in retinofugal targeting may be due to the redundancy between APP and its related family members APLP1 and APLP2, which are also expressed by RGCs (Reinhard et al., 2005; Walsh et al., 2007). In any case, the results presented here that CNTN4-mediated targeting requires APP add to growing evidence obtained in vitro (the results presented here that CNTN4 is expressed in multiple vertebrate CNS regions (Yoshihara et al., 1995) and has been shown to be important for circuit assembly in other contexts, including olfactory sensory neuron axonal convergence in the olfactory bulb (Kaneko-Goto et al., 2008), and dendritic fasciculation and laminar targeting in the chick retina (Yamagata and Sanes, 2012). CNTN4 mutations are also associated with multiple neurological disorders, including 3p-deletion syndrome, bipolar disorder, and autism spectrum disorder (ASD; Fernandez et al., 2008; Roohi et al., 2009; Kerner et al., 2011; Zuko et al., 2013). In addition, CNTN4 maps to a chromosome 3p26, which harbors a genetic link to Alzheimer’s disease (Blacker et al., 2003). Understanding the full range of ways in which CNTN4 and APP control neural circuit assembly may eventually inform better understanding of the specific defects that underlie these diseases (Zuko et al., 2011).

Circuit Alterations in CNTN4 Mutants Cause Altered Function and Behavioral Output of the AOS

One of the central goals of developmental neuroscience is to parse how specific developmental wiring steps influence circuit function and behavior. We found that loss of CNTN4 causes a dramatic decrease in the number and intensity of c-Fos+ cells in AOS targets—an indirect readout of neural activity (Omori et al., 2005). In addition, CNTN4 mutant mice displayed defects in their ability to track drifting gratings, as compared with WT controls. We interpret these defects as a reduced efficiency of the stimulus to drive NOT neurons that generate OKR behavior (Yoshihara et al., 2009; Dhande et al., 2013; Tschetter et al., 2013). The defect in horizontal tracking is especially intriguing because even though Hoxd10-GFP RGC inputs to the NOT were significantly reduced in CNTN4 mutants, whole-eye labeling revealed that RGC axons still fill this entire target. This suggests that RGCs that target the NOT in CNTN4 mutants, such as DRD4-RGCs are less efficient in driving NOT neurons—likely because they are not optimally tuned for full-field slow-moving horizontal stimuli. A non-mutually exclusive idea is the RGC axons that abnormally target the NOT are unable to establish normal functional connections with NOT target cells. This could be due to a mismatch in cell-cell recognition factors, delays in developmental timing, or other key steps of synaptic circuit assembly. Regardless, our data support the idea that axon-target matching is a crucial step leading to normal activation patterns of target neurons and the overall circuits in which they participate.

Disease and Broader Implications

Finally, although the present study was limited to analysis of optic pathway eye-to-brain connections, it is worth noting that CNTN4 is expressed in multiple vertebrate CNS regions (Yoshihara et al., 1995) and has been shown to be important for circuit assembly in other contexts, including olfactory sensory neuron axonal convergence in the olfactory bulb (Kaneko-Goto et al., 2008), and dendritic fasciculation and laminar targeting in the chick retina (Yamagata and Sanes, 2012). CNTN4 mutations are also associated with multiple neurological disorders, including 3p-deletion syndrome, bipolar disorder, and autism spectrum disorder (ASD; Fernandez et al., 2008; Roohi et al., 2009; Kerner et al., 2011; Zuko et al., 2013). In addition, CNTN4 maps to a chromosome 3p26, which harbors a genetic link to Alzheimer’s disease (Blacker et al., 2003). Understanding the full range of ways in which CNTN4 and APP control neural circuit assembly may eventually inform better understanding of the specific defects that underlie these diseases (Zuko et al., 2011).

EXPERIMENTAL PROCEDURES

Animals

Homebox D10-EGFP (Hoxd10-GFP) mice were obtained from MMRR (Dhande et al., 2013). Contactin-4-deficient mice (CNTN4−/−) were generated as previously described (Kaneko-Goto et al., 2008). β-APP knockout mice (APPβAPPΔabo) were obtained from Jax. All experimental comparisons were littermate controls. Postnatal day 0 is day of birth. All procedures carried out in accordance with institutionally approved protocols at University of California, San Diego.

Tissue Processing

Briefly, animals were overdosed and perfused with saline followed by 4% paraformaldehyde (PFA) (see Osterhout et al., 2014). Brains were post-fixed overnight (retinas were post-fixed for 2–4 hr). After sectioning on a freezing microtome or cryostat (brains, 45 μm), secondary antibodies in blocking solution as above. Primary antibodies are rabbit anti-APP (1:1,000; Calbiochem), guinea pig anti-VAChT (1:1,000; Millipore), and rabbit anti-c-Fos (1:10,000; Calbiochem).

Monocular Enucleation

Postnatal mice (P0+) were anesthetized on ice. The eyelid was parted and eye removed, taking care to limit bleeding from the ophthalmic artery. Tissue was harvested 1 week later, at P8.

Quantification of Target Area Occupied by Hoxd10-GFP RGC Axons and Total Target Volume

Retinoreceptor nuclei labeled by intracocular CTb-594 and target boundaries determined by landmark shape (see Osterhout et al., 2014). Target volume was measured by adding values from every section through the target (using the area tool in ImageJ) and multiplying that value by the thickness of the individual sections (45 μm).
**RGC Electroporation**
Mice (P0/1) were anesthetized on ice, the eyelid opened, and DNA plasmid (~0.5–1 μl pEF-CNTN4 or pCA-tdTomato) was injected by picosyringe (see Dhonde et al., 2011). Six square wave pulses (25 V, 50 msec each, 1-s gap x three pulses of each polarity) were applied with forcep electrodes. To quantify the number of electroporated axons that arborized in the NOT, Hoxd10-GFP brains were sectioned and stained for GFP and CNTN4. Axons with a secondary branch confined to the boundaries of the NOT were considered “arborizing” in the target. Statistical significance was determined using Fisher analysis (n = 20–58 axons per plasmid/genetic background condition; 10–20 mice per electroporation group).

**Single Axon Reconstruction**
At 7 days post-electroporation, brains were sectioned at 200 μm and imaged at 25x with a Zeiss 710 confocal microscope, and axons were reconstructed and analyzed with Neuro lucida.

**Co-immunoprecipitation**
Dynabeads Co-Immuno precipit in kit (Life Technologies) and protocol were used to co-immunoprecipitate APP in P8 whole-brain lysate from WT and CNTN4 invalid mice. Lysate was spun at 840 Gs for 45 min. Western blot was analyzed using guinea pig anti-CNTN4 (1:2,000; Kaneko-Goto et al., 2008) and rabbit anti-APP (1:1,000; Calbiochem) antibodies.

**c-Fos**
P20 mice were anesthetized and fitted with a small metal head post adaptor using dental cement; recovery was 2–3 days (see Dhonde et al., 2013). Mice were dark adapted for 24 hr. Head-fixed mice were presented with a sham stimulus, horizontal-drifting or vertical-drifting, bars (0.16 cycles/degree, 1.25 degrees/s) for 2 hr (Yonehara et al., 2009; Dhonde et al., 2013). Immediately after stimulation, brains were collected and processed for c-Fos. The number of c-Fos cells was measured from throughout the NOT in both groups (n = 5–6 mice per genotype for horizontal stimulus and n = 3 for vertical and sham stimulus).

**Analysis of OKR Behavior**
WT and mutant mice (P25–P30) were placed on an elevated platform surrounded by four computer monitors (see Wang et al., 2009). Each trial consisted of horizontal or vertical drifting-bar stimuli at 0.16 cycles/degree and 12 degrees/s. Each trial lasted 15 s; if the head of the mouse moved in concert with the gratings, that trial was scored as “tracked.” Each mouse was presented with 10–12 trials per day, for 3–4 consecutive days, the same time of day. Responses were averaged to generate a median percentage of trials tracked (n = 5 mice per genotype).

**Analysis of Pupil Reflex**
Mice were dark adapted for 1 hr. Infrared video of the right eye was recorded just prior to and at the finish of a 30-s trial of blue-light stimulation directed at the left eye (Güler et al., 2008) (see Sweeney et al., 2014). The percentage change in pupil area measured in ImageJ (n = 5 mice per genotype).

**Electrophysiology**
Briefly, retinas were harvested and dissected in gassed (95% O2 with gassed Ames medium heated to 33 °C, 5% CO2) Ames medium under infrared illumination and cut along the dorsal-ventral axis (Wei et al., 2010; Stafford et al., 2014) (see Dhonde et al., 2013). Only ventral pieces were used. A piece of retina was placed in a chamber and superfused with gassed Ames medium heated to 33 °C–35 °C. GFP+ ganglion cells were visualized by an attenuated mercury light passed through a GFP dichroic mirror and then targeted for recording under infrared (IR) illumination. Cells were recorded with borosilicate glass pipettes (4–6 MΩ) filled with intracellular solution containing (in mM) 120 K-methanesulfonate 10 HEPEs, 5 NaCl, 0.1 EGTA, 2 ATP-Mg2+, and 0.3 GTP-Na, titrated to pH 7.3.

Light stimuli were generated using Psychophysics Toolbox and MATLAB. Stimuli were projected onto the retina using a Dell video projector custom fitted with a UV LED (398 nm), attenuated by a 1.0 ND filter, and focused to the level of rod and cone outer segments. The wavelength of the light stimulus is equally efficient at stimulating mouse M and S cones (Borghuis et al., 2013), and stable S-cone-mediated responses can be recorded from GFP+ cells in the ventral mouse retina that have been targeted using epifluorescence (Stafford et al., 2014). Stimuli were presented over the receptive field center as a contrast pulse or drifting square-wave gratings modulated against a mean luminance. The spatial (500 μm/cycle) and temporal frequencies (1 Hz) of the gratings were near the peak sensitivity of Hoxd10-GFP RGCs (Dhande et al., 2013).

Directional preference was determined by drifting gratings in 12 directions for 4 s with an interstimulus interval of 10 s. The number of spikes obtained during a presentation of the gratings in a given direction was considered the response for that direction. Responses were normalized by the total number of spikes in all directions, and the preferred direction was the angle of the vector sum of the normalized response. The directional selectivity index (DSI) was calculated as DSI = (preferred – null)/(preferred + null), where preferred is the response in the stimulus direction closest to the preferred direction and null is the response to the stimulus 180° opposite. The tuning width was determined by fitting the cell’s response as a function of stimulus direction with the von Mises distribution; the width was defined as the full width at half height of the von Mises fit (Elston et al., 2009).

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.04.005.

**AUTHOR CONTRIBUTIONS**
J.A.O. and A.D.H. conceived of the experiments. J.A.O. and P.L.N. performed histology. J.A.O. imaged and analyzed all the data. B.K.S. recorded receptive fields and analyzed the data. J.A.O., B.K.S., Y.Y., and A.D.H. wrote the paper.

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**REFERENCES**


## CHAPTER 3 SUPPLEMENTAL DATA

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### CNTN4 protein expression in retinorecipient targets

Figure 3.S1: Specificity of CNTN4 expression in RGC subtypes and their visual targets
Figure S1: Specificity of CNTN4 expression in RGC subtypes and their visual targets (related to Figure 1).

Chart of the major RGC subtypes (listed on left) and their retinorecipient targets. Targets with dense innervation: +++, moderate innervation: ++, and sparse innervation: +. The absence of any symbol indicates a complete lack of input to that target. References for these patterns: M1 ipRGCs (Hattar et al., 2006), M2 and M4 ipRGCs (Ecker et al., 2010; Estevez et al., 2012; Osterhout et al., 2014), t-Off α RGCs (Huberman et al., 2008; 2009), On-Off DSGCs (Huberman et al., 2009; Rivlin-Etzion et al., 2011), Hoxd10 (slow-tuned AOS projecting On-DSGCs (3 subtypes) and On-Off DSGC (one subtype) (Dhande et al., 2013; and this study).

(A-G) CNTN4 protein expression in the major retinorecipient targets. CNTN4 is either absent or expressed at very low levels in retinorecipient areas other than the NOT and MTN. These targets are only innervated by Hoxd10 On-DSGCs and one subtype of slow tuned On-Off DSGC, all of which are labeled in Hoxd10-GFP mice (and see Dhande et al., 2013 and main Figures, and S3 below.)

(A, B, D) CNTN4 expression is absent from the hypothalamic suprachiasmatic nucleus (SCN, A), and ventral lateral geniculate nucleus (vLGN, B) and intergeniculate leaflet (IGL, B) olivary pretectal nucleus (OPN; D).

(C) CNTN4 is expressed at very low levels in one subregion of the dorsal lateral geniculate nucleus (dLGN), the shell (arrow in (B)). Previous studies indicate this region receives sparse input from Hoxd10-RGCs (Dhande et al., 2013; Osterhout et al., 2014). Scale = 250μm

(E-F) CNTN4 expressed in NOT and MTN; Scale = 250μm

(G) CNTN4 is expressed at low levels in superficial retinorecipient superior colliculus (SC), the upper stratum griseum superficialis (uSGS). This subregion of the SC is known to receive sparse input from Hoxd10-RGCs (Dhande et al., 2013). ISGS: lower stratum griseum superficialis; Scale = 500μm

(H) Antibody expression is specific. CNTN4 is not expressed in the NOT (or elsewhere in the brain or retina) in CNTN4−/− mice (H). Scale = 200μm

Note: these expression patterns are all consistent with the argument that CNTN4 protein is expressed by Hoxd10-RGCs that mainly target the AOS nuclei, but not by other major groups of RGC subtypes that target other retinofugal targets.
Figure 3.S2: CNTN4−/− Hoxd10-RGCs innervate the MTN and other retinorecipient targets normally, and their dendrites stratify correctly.
Figure S2: CNTN4<sup>−/−</sup> Hoxd10-RGCs innervate the MTN and other retinorecipient targets normally, and their dendrites stratify correctly (related to Figure 2).

(A-C) Hoxd10-RGC axons in the MTN of (A) wildtype, (B) CNTN4<sup>+/−</sup>, and (C) CNTN4<sup>−/−</sup> mice. Age P8 is shown. Scale = 250μm

(D-G) Whole eye binocular labeling with CTb-594 at P5 and P8 reveals similar timing and pattern of overall RGC innervation of the NOT for wildtype (D, F) and CNTN4<sup>−/−</sup> mice (E, G). P5 and P8 timepoints are shown, which correspond to when this pretectal region receives its major input from retina (see Osterhout et al., 2014 for details). Scale = 250μm

(H-K) CTb-594 (ipsilateral eye) and CTb-488 (contralateral eye) projections to the visual thalamic targets dLGN, IGL and vLGN (H,I) and to the SC (J,K) in wildtype (H, J) and CNTN4 mutant (I, K) mice. In both areas of the retinofugal pathway RGCs (as a general population) innervate retinorecipient targets and refine to the correct laminar zones, irrespective of genotype. All targets are filled completely and binocular segregation appears normal (e.g., ipsilateral eye projections to SC are in area ventral to lSGS.) Scale = 250μm

(L-S) Dendritic stratification of monostratified Hoxd10-GFP On-DSGCs (L,M,P,Q) and bistratified Hoxd10-GFP On-Off DSGCs in (N, O, R, S) wildtype CNTN4<sup>+/−</sup> (L-O) and mutant CNTN4<sup>−/−</sup> mice (P-S). Scale = 50μm. For details on these types of RGCs expressing GFP in Hoxd10 mice, see Dhande et al., 2013 and Supplemental Figure 3.
Figure 3.S3: Loss of CNTN4 does not alter functional output or receptive field properties of Hoxd10-GFP RGCs
Figure S3: Loss of CNTN4 does not alter functional output or receptive field properties of Hoxd10-GFP RGCs (related to Figure 3).

(A) Whole-cell, current-clamp recordings from a WT On (top, left) and On-Off (top, right) Hoxd10-GFP RGC in response to a 300 μm and 200 μm diameter spot respectively, presented for 1 sec, and centered on the receptive field center. Trace above the voltage trace corresponds to the timing of the light stimulus.

(B) Same as in (A) for Hoxd10-GFP RGCs in a CNTN4-/- background. The responses of the cells are normal.

(C) Summary polar plots for WT On (left) and On-Off (right) Hoxd10-GFP RGCs. Arrows represent responses from individual cells to presentations of square-wave drifting gratings in 12 different directions (see Experimental procedures). The angle of each arrow represents the directional preference of the cell and the length represents the DSI, or the strength of the directional tuning. On Hoxd10-GFP RGCs can be grouped into three types that respond to Nasal (red), Dorsal-Temporal (blue), and Ventral-Temporal (green) motion, while On-Off Hoxd10-RGCs only respond to Nasal motion.

(D) Same as in (C) for Hoxd10-GFP RGCs in a CNTN4-/- background. There is no significant difference in the angle of the directional preference of On and On-Off Hoxd10-GFP RGCs as compared to WT cells (p > 0.3 for all; Watson-Williams Test).

(E) Quantification and comparison of the DSI for On and On-Off Hoxd10-GFP RGCs. There is no significant difference between CNTN4-/- and WT RGCs (On: p = 0.3; On-Off: p = 0.7; t-test). Number of cells analyzed in parentheses. Error bars indicate SEM.

(F) Quantification and comparison of tuning width On and On-Off Hoxd10-GFP RGCs. There is no significant difference between CNTN4-/- and WT RGCs (On: p = 0.4; On-Off: p = 0.3; t-test). Error bars indicate SEM. Number of cells analyzed in parentheses (same as in E).
Figure 3.S4: Axon-target matching by non-AOS projecting RGCs appears normal
Figure S4: Axon-target matching by non-AOS projecting RGCs appears normal (related to Figure 4).

(A-D) DRD4-GFP RGC axons correctly innervate the shell region of the dLGN (A, B) and the uSGS of the SC (C,D) in both wildtype (A,C) and CNTN4<sup>−/−</sup> (B, D) mice.

(E-L) Cdh3-GFP RGC axons correctly innervate the visual thalamus (E, F), anterior pretectum OPN (G, H), posterior pretectum PPN (I, J) and central pretectum NOT (K, L) in wildtype (E, G, I, K) and CNTN4<sup>−/−</sup> (F, H, J, L) mice. All Scale = 250µm.
Figure 3.S5: App mutant Hoxd10-RGCs innervate the MTN and other retinorecipient targets normally, and Hoxd10-RGC numbers are maintained
Figure S5: APP mutant Hoxd10-RGCs innervate the MTN and other retinorecipient targets normally, and Hoxd10-RGC numbers are maintained (related to Figure 6).

(A-C) Hoxd10-RGC axons in the MTN at age P8 in: (A) wildtype APP+/+ mice, (B) APP-/- mice, and (C) APP-/- CNTN4-/- double knockout mice (C). Scale = 250μm

(D) Total number of Hoxd10-GFP RGCs in retinas of wildtype APP+/+ mice (black bar), APP-/- mice (dark grey), and CNTN4-/-, APP-/- double knockout mice (light gray; ±SEM; t-test). (n=5-6 mice per genotype).

(E-H) CTb488/594-labeled RGC axons (as in S2) in the visual thalamus (E, F) and in the SC (G, H) of control (E, G) and (F, H) APP-/- mice. Age P15 shown. Innervation patterns of these targets are normal. Note: the slightly larger ipsilateral sector of the dLGN in APP mutants is within normal range (see Jaubert-Miazza et al., 2005). Scale = 250μm

(I-K) All Hoxd10-RGCs (arrowheads, green signal, I) express APP protein (arrowheads magenta, J). (K) merge of (I and J). The broader expression of APP is expected (see main text). Scale = 100μm.
Figure 3:S6: Loss of CNTN4 results in decreased c-Fos activation in the MTN in response to
Figure S6: Loss of CNTN4 results in decreased c-Fos activation in the MTN in response to horizontal motion stimuli, but loss of CNTN4 does not alter pupil reflexes (related to Figure 8).

(A-D) c-Fos activation in the MTN of wildtype mice in response to a sham stimulus (A), vertical motion (B), or horizontal motion (C). (D) c-Fos activation in the MTN of CNTN4-/- mice in response to horizontal motion. Scale = 250μm

(E) Quantification of the number of c-Fos expressing neurons/mm³ in the MTN of wildtype (black bar) or CNTN4-/- (gray bar) mice in response to sham stimulus or vertical or horizontal drifting gratings. (±SEM; **P<0.01; t-test) (n = 3-5 mice per condition).

(F) Percent reduction in pupil area (a direct measurement of pupil constriction) in response to stimulation with high intensity or low intensity light in wildtype (black bars) and CNTN4-/- (gray bars) mice (n=5 mice per group).

(G) Number of Hoxd10-RGCs in the retina at P30
Supplemental Data References


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CONCLUSIONS

The work presented here uncovers cellular and molecular mechanisms of axon target matching in the developing visual system. Chapter 1 detailed some of the cellular mechanisms of axon target matching for RGCs. That work showed that selectivity of target innervation increases with time. The earliest RGC axons to innervate the visual pathway project to many targets before refining their projections and the latest to innervate the visual pathway project to only the correct target from the outset. Chapters 2 and 3 provided evidence for molecular mechanisms of axon target matching. That evidence included that a particular Type-II classical cadherin, Cadherin-6 (Cdh6), acts as an adhesion molecule to promote axon target matching specificity. Loss of Cdh6 results in a decrease in Cdh3-GFP RGC projections to their targets in the pretectum. Findings from Chapter 3 further elucidated the cell adhesion mechanisms by which cell adhesion can influence axon target matching. I showed that CNTN4 and APP are required for axon target specificity between AOS-RGCs and their target in the pretectum that mediates horizontal image-stabilizing eye movements, the NOT. In both CNTN4 and APP mutant mice, AOS-RGCs were unable to fill the NOT target area. While there was a loss of projections from AOS-RGCs, the target area was instead occupied by other RGC projections. However, the behavior mediated by the NOT was still impaired, indicating that the loss of a single input to a target can be detrimental to the entire circuit.

Timing and the development of the visual system

The underlying theme of these studies is developmental timing. I found that timing of innervation played a crucial role in determining the mode and mechanisms of axon target
matching. In addition, the ability of an axon to find the right targets depends on timing of adhesion or guidance molecule expression. Cdh6 mRNA was expressed in the OPN as early as P0, when many Cdh3-RGCs are innervating that target but before other types of RGCs have begun to innervate the brain. Moreover, CNTN4 protein expression in the target was downregulated in AOS-RGC axons after most RGC axons have innervated their targets, suggesting the primary function of CNTN4 takes place during target innervation. Similarly, the timing of protein expression plays an important role in the *Drosophila* visual system, where photoreceptors 7 and 8 (R7, R8) connect to distinct layers in the medulla using ubiquitously expressed Cadherin-N (Ncad) but recognition of Ncad requires temporally regulated Sequoia protein (Petrovic and Hummel, 2008). First, R8 axons express high levels of Sequoia as they innervate the medulla, and later Sequoia expression dramatically decreases in R8 axons and at the same time increases in R7 axons during the time R7 innervation into the medulla (Petrovic and Hummel, 2008).

The innervation of specific populations of RGCs likely correlates with the development of their perspective targets. For example, the NOT experiences a large increase in size during the window of innervation by AOS-RGCs; in the days prior to RGC axon innervation the NOT is much smaller and difficult to detect. There is precedence for co-dependent timing of axon innervation and target development in other systems. During hair follicle morphogenesis, successive stages of neural innervation correlate with stages hair follicle development (Peters et al., 2002). Interestingly, the first nerve fibers innervate the region of the future hair follicles before follicular development begins and in a similar manner, Cdh3-RGC axons innervate the visual pathway during a time when the natural boundaries of retinorecipient targets are not yet visible. There is also evidence to suggest that
the arrival of sensory axons can themselves induce the development of their targets. In the
olfactory system of drosophila, the pioneer olfactory axons induce the formation of the
olfactory bulb by increasing cell cycle kinetics in target precursor cells (Gong and Shipley
1995). In the drosophila visual system, photoreceptor cells secret hedgehog protein that
induces the formation of their target, specifically by inducing the lamina precursor cells to
complete their final division and undergo differentiation (Huang and Kunes, 1996). There is
still more to learn about whether sensory axons induce the development of their targets in
mammalian systems. For example, the loss of early innervating mouse RGCs (Cdh3-RGCs)
may delay or disrupt the maturation of retinorecipient targets they come in contact with
during early development.

It seems that in systems with heterogeneous cell types successive birth order and/or
axon outgrowth is a common mechanism for organizing the wiring patterns of neural circuits.
For example, in the mouse spinal chord, the birth order of premotor interneurons correlates
with their future location within the dorsal spinal cord as well as their future wiring pattern
(Tripodi et al., 2011). Premotor interneurons born early migrate to a lateral location and form
synapses with flexor motor neurons and premotor neurons born later migrate to a more
medial location and form synapses with extensor motor neurons (Tripodi et al., 2011). In
addition, a recent paper describing a computational model that analyzes the differences
between overlapping and non-overlapping timing windows for neuron birth and axon
outgrowth (all at once vs. successive stages) indicates that time windows for axon growth has
major implications for neural circuit organization (Lim and Kaiser 2015). During successive,
non-overlapping growth, the earliest born neurons made more connections than later born
neurons but later born neurons had increased efficiency than earlier born neurons (Lim and
Neurons born at the same time had homogenous connection patterns but they showed a decrease in the probability of forming synapses with increased distance traveled as compared to serial growing axons (Lim and Kaiser 2015). Although the model was used to analyze short-range connectivity, the model and the experiments presented in Chapter 1 shared similar themes. Early born Cdh3-RGC axons were able to send projections to many targets whereas later born Hoxd10-RGC axons were more efficient at innervating the correct targets. It would be interesting to adapt a similar model to long-distance networks to try to understand how timing of axon outgrowth affects synaptic connectivity in the mammalian visual system.

**Future directions**

Understanding how a single axon finds and innervates specific targets will result in a greater understanding of how neurons achieve specificity of their connections. However, this will only provide a limited understanding of development in the mammalian brain, in which most functions require not one neuron but many neurons connected in a sequence to form a poly-synaptic circuits. Future directions for the study of axon target matching should focus on mechanisms required for specific connections between multiple, sequential target structures. One can imagine at least two different scenarios: the molecular mechanisms connecting each component of a poly-synaptic circuit could be independent of one another, using distinct ligands and receptors to build each connection, or they could use a common set of mechanisms to form correct connections. In addition they may use a similar timing mechanisms to ensure selective connectivity.
Finally, two mechanisms of axon target matching have been identified here: time-dependent cellular mechanisms and molecular adhesion mechanisms. Of course there are almost certainly other mechanisms involved too for accurate axon-target matching such as repulsion. Moreover, neural activity may also play a role. Neural activity is required for correct topographic mapping within specific targets in the visual system (Debski and Cline 2002; McLaughlin et al., 2003; Pfeiffenberger et al, 2006; Triplett et al., 2009) and is also important for correct connectivity in other projection systems and circuits like the neuromuscular junction (Buffelli et al., 2004; reviewed in Tintignac et al., 2015). It remains to be determined whether activity is an important determinant in axon target matching. It is possible that postsynaptic cells can select for and detect differences in activity patterns between different subtypes of RGCs. In Chapter 3, the slow-tuned on-off direction selective RGCs (Hoxd10-RGCs) were unable to fill their target in CNTN4 mutant mice and the fast-tuned on-off direction selective RGCs (DRD4-RGCs) took up the additional space in that target. DRD4-RGCs and Hoxd10-RGCs are a similar in the sense that they both detect directional motion but DRD4-RGCs were still unable to completely rescue the loss of motion detection in CNTN4 mutant mice, suggesting that they were unable to drive the cells within the NOT as efficiently. However, DRD4-RGCs respond to faster stimuli as compared to Hoxd10-RGCs, thus it is possible that using a faster stimulus might have activated more NOT target neurons. It seems important to understand the relationship between activity patterns and the compatibility of pre- and post-synaptic partners.

**Broader implications**

Very few, if any, studies have found that defects in axon target matching result in
developmental diseases in humans. That is likely due to the lack of knowledge about the molecules and mechanisms of axon target matching as well as tools to study wiring defects in humans. It is worth noting that both CNTN4 and APP are related to serious neurological disorders in humans. Mutations in or a loss of CNTN4 is associated with autism spectrum disorder, bipolar disorder, and 3p-deletion syndrome (Fernandez et al., 2008, Roohi et al., 2009, Kerner et al., 2011, Zuko et al., 2013). One of the first known genetic causes of Alzheimer’s disease was mutated APP, which forms amyloid plaques in the brains of patients, but very little is known about the normal function of APP (reviewed in Chouraki and Seshadri, 2014). According to data presented here, it is clear that APP is also required for normal development of the CNS and loss of functional APP could lead other neurological defects (reviewed in Nicolas and Hassan, 2014). Thus, these studies presented warrant further investigation of axon target matching in the context of mental disorders.
References


