**Drosophila N-cadherin functions in the first stage of the two-stage layer-selection process of R7 photoreceptor afferents**

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Summary

Visual information received from the three types of photoreceptor neurons (R1-R6, R7 and R8) in the fly compound eye converges to the external part of the medulla neuropil (M1-M6 layers) in a layer-specific fashion: R7 and R8 axons terminate at the M6 and M3 layers, respectively, whereas lamina neurons (L1-L5) relay R1-R6 to multiple medulla layers (M1-M5). Here, we show that during development, R7 and R8 neurons establish layer-specific projections in two separate stages: during the first stage, R7 and R8 axons sequentially target to the R7- and R8-temporary layers, respectively; and at the second stage, R7 and R8 growth cones progress synchronously to their destined layers. Using a set of mutations that delete different afferent subsets or alter R7 connectivity, we defined the mechanism of layer selection. We observed that R8, R7 and L1-L5 afferents target to their temporary layers independently, suggesting that afferent-target, but not afferent-afferent, interactions dictate the targeting specificity. N-cadherin is required in the first stage for R7 growth cones to reach and remain in the R7-temporary layer. The *Ncad* gene contains three pairs of alternatively spliced exons and encodes 12 isoforms. However, expressing a single Ncad isoform in *Ncad* mutant R7s is sufficient to rescue mistargeting phenotypes. Furthermore, Ncad isoforms mediate promiscuous heterophilic interactions in an in vitro cell-aggregation assay. We propose that Ncad isoforms do not form an adhesion code; rather, they provide permissive adhesion between R7 growth cones and their temporary targets.

Key words: *Drosophila*, N-cadherin, R7

Introduction

Neural circuits of complex brains are frequently organized into parallel layers (laminae), with distinct populations of afferents innervating specific layers (Sanes and Yamagata, 1999). Such layer specificity is widely observed in vertebrate and invertebrate brains, and it probably provides a means to simplify the complexities of synaptic wiring in the central nervous system. Cell-adhesion molecules have been proposed to mediate the interactions of afferents with their synaptic targets during layer selection (Yamagata et al., 2003). In vertebrate retina, the immunoglobulin-superfamily receptors Sidekick 1 and Sidekick 2 specify layer-specific connectivity via their homophilic adhesive activity (Yamagata et al., 2002). Furthermore, the calcium-dependent adhesion receptors, the cadherins, have been proposed to match pre- and postsynaptic partners on the basis of their homophilic binding activity, synaptic localization, and regional and dynamic expression patterns in the brain (Shapiro and Colman, 1999; Yagi and Takeichi, 2000; Iwai et al., 2002). Indeed, antibody-mediated inhibition of N-cadherin caused retinal axons to innervate incorrect layers in chick tectum (Inoue and Sanes, 1997).

The *Drosophila* visual system contains projections from the eye to the brain that segregate in specific layers; the targeting of these projections is genetically hardwired (Clandinin and Zipursky, 2002; Tayler and Garrity, 2003). It has been used as a model system for studying the development of layer-specific connections. The *Drosophila* compound eye consists of approximately 800 ommatidia, each containing three types of photoreceptor neurons (R1-R6, R7, and R8) (Meinertzhagen and Hanson, 1993). The R1-R6 neurons respond maximally to the blue/green spectrum of light and connect to the first optic neuropil (the lamina), whereas the R7 and R8 are most sensitive to the ultraviolet and blue or green spectra of light, respectively, and connect to the second optic neuropil (the medulla) (Salcedo et al., 1999). The medulla is subdivided into ten layers (M1-M10) based on the terminals of the innervating afferents: the R7 and R8 axons project to the M6 and M3 layers, respectively, while the lamina neurons (L1-L5) relay R1-R6 input to multiple medulla layers (Fischbach and Ditttrich, 1989).

Genetic screens based on visual behaviors or histology identified three surface receptors, the *Drosophila* N-cadherin (Ncad; CadN – FlyBase), the receptor tyrosine phosphatases LAR, and PTP69D, that are required for R7 layer selection (Clandinin et al., 2001; Lee et al., 2001; Maurel-Zaffran et al., 2001; Newsome et al., 2000). N-cadherin and LAR are
required cell-autonomously in the R7 neurons while PTP69D might function in R7 or R8 neurons. Removing N-cadherin or LAR in the R7 neurons results in mutant R7 afferents mistargeting to the R8-recipient layer, and defects in wavelength-disruption visual behavior. LAR is required for R7 afferents to remain in appropriate target layers during development. The mechanism of action is not known for N-cadherin. The Drosophila N-cadherin has a larger complex extracellular domain that mediates homophilic interactions (Iwai et al., 1997). It is evolutionarily conserved in worms, insects, and vertebrates (Broadbent and Pettitt, 2002; Tanabe et al., 2004), and thus may be the most ancient form of classic cadherins. In this study, we investigated the developmental processes of medulla layer formation and the role of N-cadherin therein.

Materials and methods

Genetics

Gcm-Gal4 driver (a generous gift from Iris Salecker) is active in lamina precursor cells, and was used to label young L1-L5 axons. For labeling R7s at various developmental stages, the following drivers and genetic schemes were used: PM181-Gal4 driver, which is expressed in R7s prior to axonogenesis, has been described previously (Lee et al., 2001). The relative times of arrival of R7 and L1-L5 axons at the medulla were approximated based on the appearance of the markers in the medulla. UAS-Gal4 (Hasan et al., 2000) was used to extend the activity of PM181-Gal4 for labeling R7 axons at 35% APF (after puparium formation). UAS-mCD8-GFP or UAS-lacZ was used with various Gal4 drivers to label different types of afferents. To label R7 axons beyond 40% APF, we used PM181-Gal4 to drive UAS-Flp. The expression of flipase in R7s removes the interruption cassette in the GMR<interrupt>GFP transgene (a gift from Paul Garrity), and the GMR promoter, in turn, drives the expression of a membrane-tethered GFP (myr-GFP) in the R7s at the late pupal stage. For labeling adult R7s, R7-specific opsin drivers, Rh3-Gal4 and Rh4-Gal4 were used; for adult R8s, Rh5-Gal4 and Rh6-Gal4, were used; for adult L2 cells, L2-Gal4, kindly provided by Andreas Keller, was used; for medulla neurons, Apterous-Gal4 was used.

For genetic cell-ablation experiments, hh1 (eye-specific hedgehog allele (Huang and Kunes, 1996), a gift from Sam Kunes), used: (1) FRT40; (2) Ncad<sup>405</sup> FRT40/Cyo, ubiP-GFP; (3) Ncad<sup>417</sup> FRT40/Cyo, ubiP-GFP; (4) Elav-Gal4<sup>155</sup>, UAS-mCD8-GFP, hs-Flp; tubP-Gal80 FRT40.

Histology

Immunohistochemistry was performed as described previously (Lee et al., 2001). Confocal images were acquired using a Zeiss 510 META laser scanning microscope. The obtained z-stacks of images were deconvolved to remove out-of-focus light and z-distortion with Huygens Professional software (Scientific Volume Imaging) running on a 48 processors SGI Origin 2000. The 3D images were rendered from the restored z-stacks using Imaris software (Bitplane).

Ncad genomic sequence and transcript analyses:

Blast searching using Bioperl scripts (Stajich et al., 2002) and web-based servers was performed to identify potential alternative exons, which were further examined for appropriate splicing donor and acceptor sites. The Ncad cDNA corresponding to the 7b-13a-18b isoform (Iwai et al., 1997) was used as query to search against the genomic sequences of Drosophila melanogaster (Adams et al., 2000), Drosophila pseudoobscura, Anopheles gambiae (Holt et al., 2002), andApis mellifera. In addition to the exons 7b, 13a, and 18a reported previously (Iwai et al., 1997), these analyses identified three potential exons corresponding to the exons 7a, 13b, and 18b (Fig. 5).

To obtain experimental evidence for Ncad alternative splicing, we analyzed Ncad transcripts using RT-PCR. The regions containing potential alternative exons were amplified using the Titan One tube RT-PCR System (Roche), subcloned into the pCR 2.1 vector using the TOPO cloning system (Invitrogen), and sequenced. The obtained cDNA sequences matched the exon sequences predicted from genomic sequence analysis. In addition, a small insertion of 12 bp was found in approximately half of the cDNAs that contain exon 7a. This sequence was further mapped to a small exon, designed 7a′, that is located between the common exon 6 and the alternative exon 7a. The sequences of the primers used in RT-PCR are available upon request.

Molecular biology

The Ncad isoform 7b-13a-18a and E-cadherin S2 expression vectors and cDNAs were generous gifts from Tadashi Uemura (Iwai et al., 1997; Oda et al., 1994). The pRmHa3/Ncad vectors, for expressing different Ncad isoforms in S2 cells, and the pUAS-Ncad vectors, for transgene rescue experiments, were constructed as follows. The variable regions corresponding to exons 7a, 13b, 18b were cloned into the S2 expression vector, pUAST. Transgenic flies were generated using standard microinjection techniques.

Cell-aggregation assay

For expressing different Ncad isoforms in suspension S2 cells, different Ncad isoform expression vectors (pRmHa3/Ncad) were cotransfected with a GFP or Ds-red expression vector (pRmHa3/GFP and pRmHa3/Ds-red). Suspension S2 cells were a generous gift from James Clement (Schmucker et al., 2000). Cell culture and transfection were performed according to the Invitrogen DE3 and Qiagen Effectene manuals. CuSO4 was added (0.7 µM) 24 hours after the transfection to induce the expression of Ncad isoforms and the GFP or Ds-red marker. The S2 cells were induced for 48 hours and then subjected to cell-aggregation assay as described previously by Oda.
Development

R7 target selection is a two-step process

(Oda et al., 1994) except for the following modifications. Two populations of S2 cells expressing different cadherins and markers at the concentration $1.2 \times 10^6$ cells/ml were incubated in 2 ml of the BBS buffer (mM: 10 Hepes, 55 NaCl, 40 KCl, 15 MgSO4, 20 glucose, 50 sucrose) containing 5 mM of CaCl2 in a 35 mm polystyrene dish, and agitated using a gyratory shaker at 100 rpm for 1.5 hours. The formation of cell aggregates was analyzed under a Zeiss M'bio fluorescence microscope with a Zeiss AxioCam digital camera. Experiments were performed in triplicates.

Western blot

Cell lysis and immunoblotting were performed as described (Lee et al., 2001). For each lane, a protein sample equivalent to 200,000 S2 cells was loaded. Rat monoclonal antibody against the cytoplasmic domain of Ncad proteins (Iwai et al., 1997) (a gift from Tadashi Umura) was used to detect the Ncad proteins.

Results

R7 target selection occurs in two distinct stages

To gain insight into the developmental mechanisms governing the formation of layer-specific connections within the medulla, we examined the innervation of medulla by R7, R8, and L1-L5 afferents at various developmental stages. The R-cell growth cones begin to segregate into discrete layers at ~17% pupal formation (17% APF; eclosion at 100%) (Fig. 1A,A'). During this stage, there is a gradient of R7, R8, and L1-L5 growth cones in the medulla neuropil along the anterior-posterior axis, with the newly arriving axons projecting into the anterior edge of the medulla and the older growth cones fully expanded in the posterior edge (Fig. 1A'.B'.B'). This growth-cone gradient reflects the developmental sequence of the eye discs and the lamina (Fig. 1E, black arrows.)

Superimposed on this temporal gradient is a developmental sequence of the R-cell subtypes and the corresponding lamina neurons (L1-L5). Within each ommatidium, R8 differentiates first, induces the differentiation of the remaining R-cells and L1-L5, and extends its axons into the superficial medulla (R8-temporary layer, Fig. 1A''). The R7s differentiate approximately 24 hours after R8s, and they project axons past the R8 growth cones and terminate at a layer below (R7-temporary layer, Fig. 1A'') (Lee et al., 2001). Based on the R7 (PM181-GFP) and L1-L5 (Gcm-LacZ) markers, we estimate that approximately 2-4 hours after the arrival of R7 growth cones at the medulla (corresponding to 1-2 rows), L1-L5 axons reach the medulla and terminate between the R7 and R8 growth cones (Fig. 1B''). The L1-L5 axonal projections in the medulla were further confirmed with the MARCM (mosaic analysis with a repressible cell marker) system (Lee and Luo, 1999), which allowed us to trace single L1-L5 axons (see Fig. S1 in the supplementary material and data not shown). Thus, the layer-specific targeting of R7, L1-L5, and R8 (positioned from proximal to distal layers) at 17% APF does not arise simply from the order of their innervation (R8 first, then R7, and lastly L1-L5), but probably reflects specific properties of different afferents. The distance between R7 and R8 layers increases from ~7 µm at 17% APF to ~10 µm at 40% APF as the intervening layers further divide into four layers (Fig. 1D') (C.-Y.T. and C.-H.L., unpublished). However, the relative positions of R7, L1-L5, and R8 growth cones (from proximal to distal layers) remain as such until 50% APF, when these growth cones progress to reach their final target layers.

The second stage of R7 and R8 target selection starts at approximately 50% APF. Because the R7-specific Gal4 driver, PM181-Gal4, is inactive at this stage, we used a flipase-based system to label late R7 axons (see Materials and methods). We observed that at this stage the R8 growth cones projected past the L2 growth cones to reach the R7-temporary layer, which later became the R8-recipient layer (Fig. 2B',B'). In addition, the R7 axons extended approximately 3-5 µm further into the medulla. During this period, it is possible that as the medulla neuropil continued to develop (see Fig. S2 in the supplementary material), the R-cell growth cones might progress from their temporary layers to their destined layers by processes of active migration and/or passive displacement by the ingrowing medulla processes (or L4-L5 growth cones). By 70% APF, the R7 and R8 growth cones reached their final layers and assumed their adult configurations (Fig. 2D,D').

Thus, layer selection by R7 and R8 axons occurs in two stages. During the first stage, newly differentiating R8 and R7 axons reach their temporary target layers in temporal order according to their time of birth. During the second stage, the R7 and R8 growth cones progress synchronously to their final target layers (Fig. 2H).

R8, R7, and L1-L5 axons target to distinct medulla layers independently at the first layer-selection stage

Because the projections of L1-L5 axons into the medulla coincide with the separation of the R7 and R8 growth-cone layers, we addressed whether proper medulla lamination requires afferent-afferent interactions, using mutant animals lacking L1-L5 or R7s. In hh$^{\text{fr}}$ (an eye-specific allele of hedgehog) mutant animals, which lack L1-L5 (Huang and Kunes, 1996; Poeck et al., 2001), we observed that the R7 and R8 growth cones still formed two layers in the medulla although they remained closely associated during the early pupal stage (17% APF; Fig. 3A',A'). To confirm this result with a different approach, we blocked L1-L5 differentiation using Gem-Gal4 to drive the expression of EGF$^{\text{DN}}$, a dominant negative form of the EGF receptor, in the lamina precursor cells (Huang et al., 1998) (see Materials and methods). This manipulation blocks the differentiation of younger L1-L5 and recapitulated the hh$^{\text{fr}}$ phenotype in the younger part of the medulla (bracket in Fig. 3C'). Thus, L1-L5 are not required for the layer selection of R7 and R8 afferents; rather, the L1-L5 growth cones intercalate between the R8 and R7 growth cones to separate them into two distinct layers during the first layer-selection stage (17% APF). Furthermore, in hh$^{\text{fr}}$ mutants, the R8 and R7 growth cones segregate into two separate layers at 40% APF (Fig. 3B',B'), presumably due to the extension of intervening dendritic processes of medulla neurons at this stage (C.-Y.T. and C.-H.L., unpublished). Thus, L1-L5 are not specifically required for the separation of the R7 and R8 growth cones.

Conversely, in sevenless mutants, which lack R7 neurons (Basler and Hafen, 1989), we observed that L1-L5 and R8 growth cones target to the correct layers (Fig. 3D,D') at 17% APF, indicating that R7s are not required for R8 and L1-L5 axons to reach their temporary target layers. Thus, the interactions between R8, R7, and L1-L5 afferents play only a minor role in medulla layer formation, and afferent-target interaction probably dictates the layer specificity.
N-cadherin is required for R7 axons to reach and remain in the R7-temporary layer

Using the ultraviolet/visible (UV/Vis) light choice test, we have previously demonstrated that R7 layer selection requires N-cadherin (Lee et al., 2001). To determine the developmental stage during which Ncad functions and to identify the developmental defects in Ncad mutant R7s, we modified the single-cell mosaic method previously used for adult phenotypic analysis (GMR-Flp/MARCM), by including the pan-neuronal driver, Elav-Gal4. This method allowed us to analyze single...
R7 target selection is a two-step process

Fig. 2. R8 and R7 growth cones reach their destined layers during the second layer-selection stage. (A–D’) R7 and R8 layer-specific targeting assessed at 40% (A, A’), 50% (B, B’), 60% (C, C’), and 70% APF (D, D’). Approximately 70% of R7 axons were labeled using a flipase-based method (see Materials and methods) and visualized with the anti-GFP antibody (green). R8 axons (red) were marked with RFP but not GFP. The neuropils were stained with the anti-HRP antibody (purple). (A’) At 40% APF, R7 (yellow, large arrow) and R8 (red, large arrowhead) growth cones terminate at their temporary layers. (B’) At 50% APF, R7 growth cones (yellow, large arrow) extend ~3 µm further with filapodia (small arrow) leaving behind in the R7-temporary layer. R8 growth cones (red) extend filapodia (small arrowhead) deeper into the medulla while leaving their growth cone proper (large arrowhead) in the R8-temporary layer. (C’) At 60% APF, both R8 (large arrowhead) and R7 (large arrow) growth cones have reached their final target layers. Note that R7 axon shafts expanded in the R7-temporary layer (small arrow). (D’) At 70% APF, both R8 (large arrowhead) and R7 growth cones retract their filapodia and consolidate into axonal terminals. Two types of R7 terminals, straight (large arrow) and horseshoe (double-arrow) are noticeable (as seen in adult flies). (E–G’) Layer-specific connections of R7 (C, C’), R8 (D, D’) and L2 (E, E’) assessed at the adult stage. (E, E’) R7 axons (green) were labeled with Rh3 Rh4-Gal4, UAS-synb-GFP. Glass-lacZ marker labeled both R7 and R8 axons (red). (F, F’) R8 axons (green) were labeled with Rh5 Rh6-Gal4, UAS-synb-GFP. (G, G’) L2 axons (green) were labeled with L2-Gal4 UAS-mCD8-GFP. R7 and R8 axons were visualized with Mab24B10. Note that L2 axons terminate at the M2 layer, just above the R8-recipient layer (M3). (H) A schematic diagram showing the progression of R7 and R8 axonal targeting during the second layer-selection stage. For clarity, most L1–L5 neurons are omitted in the figure for the pupal stages. Abbreviations as in Fig. 1.
wild-type or mutant R7 axons up to 35% APF during which the Elav driver is active. We observed that at 17% APF, 21% (n=43) of the Ncad mutant R7 growth cones failed to reach the R7-temporary layers (Fig. 4B,B′). In addition, many (63%, n=43) Ncad mutant R7 growth cones exhibited various morphological defects: they failed to expand fully in the medulla, some expanding prematurely before reaching the appropriate layer. We next examined the Ncad mutant phenotype at 35% APF, during which the wild-type R7 and R8 growth cones formed two separate layers (Fig. 4E,E′). We observed that 55% (n=26) of the Ncad mutant R7 axons failed to target to the R7-temporary layer; instead, they terminated at the R8 layer or the layer between the R7 and R8 layers (Fig. 4F,F′). Similar phenotypes and expressivity were observed for three Ncad alleles, Ncad<sup>405</sup>, Ncad<sup>M19</sup>, and Ncad<sup>b11</sup> (data not shown). We conclude that at least 24% of the Ncad mutant R7 growth cones reach the R7-temporary layer at 17% APF, but retract from the correct layer at 35% APF (results summarized in Fig. 6E).

In contrast, LAR mutant R7 axons target correctly at both 17% and 35% APF (arrows, Fig. 4C,C′,G,G′), although some mutant growth cones exhibit collapsed morphology (arrowheads, Fig. 4G′). Using eye-specific mosaic analyses, previous studies showed that the LAR mutant R7 growth cones retracted to the R8-temporary layer in the older part of the medulla (Clandinin et al., 2001; Maurel-Zaffran et al., 2001). However, the single-cell mosaic method cannot be used to analyze the older R7 axons because the Elav-Gal4 driver becomes inactive in these neurons. Removing both Ncad and LAR in single R7 axons resulted in similar phenotypes to those seen in Ncad mutants (Fig. 4D,D′,H,H′). In summary, Ncad, but not LAR, is required for R7 axons to project into the appropriate layer and to remain in the R7-temporary layer at the early pupal stage (17-35% APF).

Because Ncad can function as homophilic adhesion molecules in vitro (Iwai et al., 1997) (see below), we next examined whether R7 growth cones use Ncad to interact with R8s or medulla neurons, both of which express Ncad (Lee et al., 2001). Using the MARCM system, we generated Ncad mutant R8 and medulla neuron clones and assessed layer selection in the corresponding R7 growth cones. Removing Ncad in single R8 neurons caused R8 growth cones to detach from the R8-temporary layer (62%, n=13), but had no effect on the R7 growth cones. Therefore, Ncad is required for R7 axons to target correctly into the appropriate layer and to remain in the R7-temporary layer at the early pupal stage (17-35% APF).
The N-cadherin gene undergoes alternative splicing to generate multiple isoforms

The N-cadherin gene contains three exon modules corresponding to exons 7, 13, and 18, each of which is composed of a pair of highly similar but distinct exons, designated exons 7α/7β, exons 13α/13β, and exons 18α/18β (Fig. 5) (see http://www.flybase.org/bin/bidq.html?FBrf0141810). RT-PCR analysis further revealed that mature Ncad transcripts contain one and only one of the two alternative exons arising from each exon module (i.e. exon 7α or 7β; 13α or 13β; 18α or 18β), indicating that these exons are used...
Fig. 5. Modular exon organization of the Ncad gene. The Ncad gene contains 21 exons that span approximately 100 Kb of genomic DNA. Mutually exclusive alternative splicing occurs in exons 7, 13, and 18. As deduced from cDNA sequences, mature mRNA contains exon 7a or 7b, exon 13a or 13b, and exon 18a or 18b. In addition, exon 7a′ is found in some exon 7a-containing transcripts. Constant exons are shown as red boxes and alternative exons as green (7a, 13a, 18a) or blue (7b, 13b, 18b) boxes. Exons 7a and 7b each encode the C-terminal half of CA8 and the N-terminal half of CA9; exons 13a and 13b each encode the C-terminal half of CA11 and the N-terminal half of CA12; exons 18a and 18b each encode the C-terminal half of EGF-CA3, the entire EGF-CA4, and the N-terminal half of the TM. The exon 7a′ encodes an insertion of four amino-acid residues in CA8. The amino-acid sequence identity between the regions encoded by the alternative exons is indicated. SP: signal peptide; CA: cadherin domain; EGF: EGF-like calcium-binding repeat; LamG: Laminin-G-like domain; TM: transmembrane region; CP: cytoplasmic domain containing β-catenin binding site.

Expressing single Ncad isoforms is sufficient to rescue R7 targeting defects of Ncad mutants

Because the developing eye discs express multiple Ncad isoforms, we carried out transgene rescue experiments to address whether multiple Ncad isoforms are required for R7 targeting. In these experiments, we combined the GMR-Flp/MARCM system (Lee et al., 2001; Lee et al., 2000) with the UAS-Ncad isoform transgene to express a single type of Ncad isoform in Ncad mutant R7s. We found that expressing a single Ncad isoform, including 7a-13a-18a, 7b-13a-18a, 7b-13b-18a, and 7b-13a-18b, substantially, if not completely, rescues the Ncad phenotypes in R7 axons (Fig. 6A-E, and data not shown). We found no significant difference in the abilities of different Ncad isoform transgenes to rescue the mistargeting or growth cones morphological defects at either 17% or 35% APF (Fig. 6E).

We next sought to determine whether in the wild-type background, mis- or over-expressing a single Ncad isoform in R7s alters their target specificity. We used the R7-specific Gal4 driver, PM-181-Gal4, to express Ncad isoforms in the wild-type background. We found that expressing any of the isoforms in R7 axons caused modest mistargeting and growth-cone morphological defects (see Fig. S5A′,B′ in the supplementary material). However, both defects were reduced in the older R7 axons (bracket, see Fig. S5A′′,B′′ in the supplementary material) and were not observed at the later stage (data not shown). These findings indicate that overexpressing single Ncad isoforms in R7 neurons is insufficient to permanently change the R7 target specificity.

N-cadherin isoforms mediate promiscuous heterophilic interactions

Iwai and colleagues previously reported that the Ncad isoform 7b-13a-18a is capable of mediating homophilic interaction in a calcium-dependent manner (Iwai et al., 1997). Because the alternative isoforms differ in the extracellular and transmembrane regions, which could potentially affect the binding specificity, we determined whether the in vitro adhesive activity of Ncad isoforms correlates with their indiscriminating activity in R7 layer selection. We modified the S2 cell-aggregation assay to test whether Ncad isoforms can mediate heterophilic interaction with one another and with another Drosophila classic cadherin, E-cadherin (Oda et al., 1994). Two populations of S2 cells that expressed different types of Ncad isoforms were labeled separately with GFP and Ds-Red and mixed using a gratory shaker under constant rotating speed (see Materials and methods for details). The expression levels of different Ncad isoforms were found to be similar (see Fig. S6 in the supplementary material). We found that all tested Ncad isoforms induced mixed-cell aggregates, indicating that they can mediate heterophilic interactions (Fig. 7B-E). However, the Ncad-expressing S2 cells did not intermix with the S2 cells expressing E-cadherin; instead, they formed separate cell aggregates (Fig. 7A). In summary, the tested Ncad isoforms are capable of mediating type-specific heterophilic interactions, but they fail to show any detectable isoform specificity in the in vitro cell-aggregation assays.
Discussion

In this study, we reveal that R7 layer-specific targeting occurs in two distinct stages: at the first stage, R7 afferents target to the R7-temporary layer where they remain for about one day until the mid-pupal stage (50% APF) when the R7 growth cones progress synchronously to their final target layer. The two-step target selection has been observed in the vertebrate hippocampus: during embryonic development, entorhinal axons and commissural and associational axons form transient synapses with Cajal-Retzius cells and GABAergic interneurons, respectively, before they synapse onto their postnatal targets, the pyramidal neurons (Bayer and Altman, 1987; Super and Soriano, 1998). The two-stage R7 layer selection might serve to coordinate afferent innervation with target development, as in the hippocampus. Alternatively, it might function to reduce the number of potential targets among which R7 growth cones must choose.

Using the mutations that delete different afferent subsets or alter R7 connectivity, we defined the mechanism of R7 layer selection. The genetic cell-ablation results suggest that R8, R7, and L1-L5 afferents target to their temporary layers independently. In addition, the wild-type R8 axons target correctly when the neighboring Ncad or LAR mutant R7s mistarget to the R8-recipient layer (Fig. 4J’,K’). Conversely, the removal of Ncad in single R8s disrupts R8 targeting without affecting the targeting of the neighboring R7s. Thus, the first stage of medulla layer-selection by R8, R7, and L1-L5 afferents probably involves primarily afferent-afferent interactions. In contrast, R1-R6 growth cone sorting to different lamina cartridges involves both afferent-afferent and afferent-target interactions (Clandinin and Zipursky, 2000; Meinertzhagen and Hanson, 1993), even though it required Ncad and LAR (Lee et al., 2001; Clandinin et al., 2001).

Developmental analyses of single Ncad mutant R7s revealed that Ncad is required for R7 axons to reach and to remain in the R7-temporary layer during the first layer-selection stage. The medulla contains over 50 different types of neurons (Fischbach and Dittrich, 1989) and many express Ncad during development. It is not technologically feasible at the current stage to remove Ncad activity in all or a large number of medulla neurons without affecting the pattern of the optic lobe. Thus, even though we found that removing Ncad in small patches of medulla neurons did not affect R7 layer selection,
we cannot rule out the possibility that multiple medulla neurons provide redundant N-cadherin-mediated interactions for R7 growth cones in a similar fashion as L1-L5 neurons do for R1-R6 afferents (S. Prakash and T. Clandinin, personal communication). Alternatively, Ncad might function as a signaling receptor, rather than as a passive adhesive molecule in R7s. Recent studies demonstrated that the cytoplasmic domains of vertebrate classic cadherins could regulate the actin-cytoskeleton via catenins (Yap and Kovacs, 2003). The *Drosophila* Ncad contains the two conserved cytoplasmic regions that interact with catenins in vertebrate cadherins. It is conceivable that Ncad could regulate the actin-cytoskeleton in R7 growth cones in response to target-derived cues. Furthermore, although Ncad and LAR share the same adult phenotype, their differential onset of mutant phenotype and double mutant phenotype suggest that they probably regulate different aspects of the first R7 layer-selection stage. Ncad is required for R7 growth cones to initially target to, and remain in the R7-temporary layer throughout the first target-selection stage, while LAR is only required during the later phase (Clandinin et al., 2001; Maurel-Zaffran et al., 2001).

The comparison between the first and second stages of R7 layer selection reveals a glimpse of the underlying mechanism. First, targeting to the R7-temporary layer at the first stage appears to be critical for the R7 axons to reach their final destination. Ncad or LAR mutant R7 axons that mistarget to the R8-temporary layer at the first stage, later proceed to terminate incorrectly as well at the R8-recipient layer. Second, in contrast to the initial target selection which follows the axon outgrowth, all R7 and R8 axons enter the second layer-selection stage at approximately the same time, regardless of when they arrive at the medulla. Interestingly, centripetal growth of R1-R6 terminals and synaptogenesis in the lamina coincide with the second stage of R7 layer-selection (Meinertzhagen et al., 2000). It is tempting to speculate that a global signal triggers the initiation of the second stage.

In this study, we report that the *Ncad* gene in *Drosophila*, and probably in other insects, undergoes alternative splicing to generate multiple isoforms. However, the lack of isoform-specificity, revealed by the transgene rescue, overexpression experiments, and heterophilic interaction assays, argues against the hypothesis that the Ncad isoforms constitute an adhesion code to direct targeting specificity. Instead, we favor the idea that Ncad plays a permissive role in R7 layer selection. Nevertheless, the remarkable conservation of the Ncad alternative splicing over 250 million years of evolution suggests an adaptive advantage for Ncad molecular diversity, whose function awaits further investigation.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/5/953/DC1

**References**


R7 target selection is a two-step process