Cadherin is required for dendritic morphogenesis and synaptic terminal organization of retinal horizontal cells

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INTRODUCTION

During development, neurons extend their dendritic processes within particular areas to receive axonal inputs. The branching patterns of the dendrites are precisely regulated and a number of molecules control the morphogenetic processes of dendrites (reviewed by Cline, 2001; Jan and Jan, 2003; Kim and Chiba, 2004; Scott and Luo, 2001; Whitford et al., 2002). Studies investigating the mechanism of dendrite formation at the molecular level have largely relied on technologies that enable the reproducible visualization of dendritic morphology of particular neurons and the simultaneous manipulation of gene functions in the same cells (reviewed by Scott and Luo, 2001; Jan and Jan, 2003). Hence, most of the in vivo analyses have been carried out in invertebrates such as Drosophila, an organism that allows advanced molecular and genetic manipulations (reviewed by Scott and Luo, 2001; Jan and Jan, 2003). As for higher vertebrate species, our knowledge is largely limited to that obtained from in vitro culture systems (reviewed by Whitford et al., 2002). Therefore, a model system that enables us to analyze the molecular mechanisms of dendritic morphogenesis directly in vivo has been awaited. We accordingly explored an alternative higher vertebrate model system that allows us to induce the expression of exogenous genes in particular neurons and simultaneously visualize the fine morphology of these cells.

Among the molecules known to control dendritic morphogenesis, we have been focusing on the cadherin family of adhesion molecules. Cadherins mediate Ca2+-dependent cell-cell adhesion, and endow cells with homophilic adhesiveness in a subtype-specific manner (Takeichi, 1995). Immunoelectron microscopic studies have revealed that cadherins, as well as their cytoplasmic regulator catenins, are localized at synaptic junctions (Uchida et al., 1996; Fannon and Colman, 1996). In cultured hippocampal neurons, cadherins control the morphogenesis and stability of dendritic spines (Abe et al., 2004; Okamura et al., 2004; Togashi et al., 2002; Tanaka et al., 2000). In hippocampal slice cultures, cadherins also regulate synaptic plasticity during LTP (Tang et al., 1998; Bozdagi et al., 2000). All of these studies strongly support the view that cadherins regulate synapse formation in the nervous system (reviewed by Shapiro and Colman, 1999; Takeichi and Abe, 2005; Uemura, 1998). Drosophila N-cadherin (DN-cadherin)-deficient mutants show a variety of abnormalities, including aberrant fasciculation and misrouting of axons in their larval central nervous system (Iwai et al., 1997). In DN-cadherin mutant allele-bearing flies that survive to the adult stage, the synaptic architecture of their nerve terminals is disorganized (Iwai et al., 2002). Mosaic analysis of mutant cells also revealed that DN-cadherin is required for correct target selection and synapse formation of photoreceptor cell axons (Lee et al., 2001; Nern et al., 2005; Prakash et al., 2005; Ting et al., 2005). DN-cadherin also controls refinement of the glomerulus-specific dendrite projection of olfactory projecting neurons (Zhu and Luo, 2004). As for vertebrate studies, N-cadherin mutant mice or fish exhibit gross abnormalities in their early neuroepithelial structures (Erdmann et al., 1997; Lele et al., 2002; Malicki et al., 2003; Masai et al., 2003; Radice et al., 1997), which subsequently cause a number of secondary effects on neuronal morphogenesis. Therefore, it is essential to manipulate cadherin activity in a cell type- and stage-specific manner to fully address its functions in the nervous system.

In the present study, we investigated the role of cadherins in the dendritic morphogenesis of horizontal cells in the developing chicken retina. To do this, we introduced a novel, transposon-mediated gene transfer system that enables conditional expression of exogenous genes. We show that the perturbation of cadherin function decreased the dendritic field size of horizontal cells, while leaving axonal elongation unaffected. We also show that cadherin was necessary for the proper termination of dendritic processes onto photoreceptor cells, as well as for the accumulation of synaptic markers at their contact points. We thus provide compelling

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evidence showing that cadherin is required for dendrite morphogenesis and synapse formation in the developing vertebrate nervous system.

**MATERIALS AND METHODS**

**Vector constructions**

Vectors were constructed as follows.

- pT2K-CAGGS: the EF promoter of pT2KXIG (Kawakami et al., 2004) was replaced by the CAGGS promoter (Niwa et al., 1991).
- pT2K-rTA-M2: cDNA fragment of M2, a modified tetracycline-responsive activator, was excised from rTA-M2 (Urlinger et al., 2000) and subcloned into the pT2K-CAGGS.
- pT2K-BI-mEGFP: the EF promoter of pT2KXIG was removed and replaced by the expression cassette derived from pBI-EGFP (CLONTECH), which contains TRE, two minimal promoters of CMV, and poly A signals; subsequently, the EGFP insert was replaced with mEGFP (generated by Ichii Tetsuo, RIKEN CDB, Japan) that contains a membrane localization signal of GAP43 (Okada et al., 1999) at N’ terminal.
- pT2K-BI-mEGFP-myc: the myc-tagged cassette from pCS2+MT (a kind gift from D. Turner) was excised and subcloned into pT2K-BI-mEGFP.
- pT2K-BI-mEGFP-cNcad-myc and pT2K-BI-mEGFP-cN390Δ-myc: coding sequences without stop codon of full-length N-cadherin (cNcad) and cN390Δ (Fujimori and Takeichi, 1993) were amplified by PCR and subcloned into pT2K-BI-mEGFP-myc.

For generating the cRNA probe of N-cadherin, we amplified the cDNA fragment corresponding to 1149-1991 of the coding sequence by PCR and subcloned it into pCRII (Invitrogen).

**In ovo electroporation**

In ovo electroporation was performed as previously described (Kubo et al., 2005). For co-electroporation experiments, plasmids at the same concentration were mixed at the same ratio (1:1:1 or 1:1). For inducing the expression of GAP43 (Okada et al., 1999) at N terminal.

**Tissue preparation and immunohistochemistry**

The retinas were dissected and fixed for 1 hour at room temperature in 4% paraformaldehyde in saline buffered with HEPES (10 mM, pH=7.4). After the retinas had been washed three times in TBS supplemented with 1 mM CaCl2 (TBS-Ca), the associated pigment epithelium was carefully removed, and retinal fragments of 0.5 cm² were dissected from the central region of the retina devoid of the optic nerve head. This region was kept constant for all analyses. The explants were incubated for 1 hour in a blocking solution containing 5% fetal calf serum (FCS) in TBS-Ca supplemented with 0.5% Triton-X 100 and 0.04% sodium azide (TBST-Ca). They were subsequently incubated for 3 days at 4°C with the primary antibodies diluted in the blocking solution, and thereafter washed three times with TBST-Ca (30 minutes each time). The samples were then incubated in the blocking solution for 30 minutes followed by 1 day in a secondary antibody solution, and then washed again three times for 20 minutes each time with TBST-Ca. For section immunostaining, the fixed retinas were cryoprotected in 20% sucrose/TBS-Ca, embedded in OCT compound (Tissue-Tek), and sectioned at 16 μm using a cryostat. The sections were mounted on APS-coated glass slides (Matsunami, Japan) and processed using a standard protocol. In situ hybridization and double immunostaining was carried out according to the protocol previously described (Tanabe, 2004). In brief, after washing in situ probes, the sections were incubated in a mixture of anti-Prox1 antibody and alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche) for 1 hour, washed three times for 20 minutes each time with TBST-Ca, and subsequently incubated with Alexa488-conjugated anti-rabbit IgG. After washing the secondary antibody, in situ hybridization signals were visualized with HNPP-FastRed (Roche) according to the manufacturer’s instructions and immediately observed under the fluorescent microscope. The antibodies used were the following: rat monoclonal anti-GFP (1:1000, clone GF090R, Nakalaitesque, Japan), mouse monoclonal anti-myc (1 μg/ml, clone 9E10, Developmental Studies Hybridoma Bank), rabbit anti-myc (1:500, Sigma C3956), rabbit anti-N-cadherin (1:1000) (Matsunaga et al., 1988), rabbit anti-Prox1 (1:500, AB5475, Chemicon), rabbit anti-GluR4 (1 μg/ml, AB1508, Chemicon), mouse monoclonal anti-Pax6 (partially purified, 1:200, Developmental Studies Hybridoma Bank), Alexa 488-

**Fig. 1. A transposon-mediated gene transfer system that enables conditional gene expression in the developing chicken retina.**

(A) Schematic drawing of conditional gene expression. The gene cassettes flanked by the Tol2 cis-sequence (Tol2) are integrated into the host genome by the transient expression of Tol2 transposase (T2TP). Only the cells that stably incorporated both CAG-rTA-M2 and TRE-mEGFP cassettes express mEGFP upon the addition of doxycycline (DOX), which enhances the binding of rTA-M2 to its cis-element TRE. (B, C) Exogenous mEGFP expression (green) in horizontal cells expressing Prox1 (B) and Pax6 (C) (magenta). Scale bars: 20 μm.
conjugated anti-rat IgG (0.5 μg/ml, Invitrogen), Cy3-conjugated anti-mouse IgG (0.5 μg/ml, Chemicon), Alexa 647-conjugated anti-rabbit IgG (0.5 μg/ml, Invitrogen), biotinylated peanut agglutinin (1 μg/ml, B1075, Vector Laboratory) and Alexa 594-conjugated streptavidin (0.5 μg/ml, Invitrogen).

Image capturing and statistical analysis

The retinal explants were mounted scleral side up in FluorSave (Calbiochem) on a glass slide. Confocal images were obtained using a confocal microscope, LSM-PASCAL or LSM510 (Zeiss), with a 63×/1.2NA or 40×/1.2NA water immersion objective (Zeiss). Using a LSM Image Analyzer (Zeiss), three-dimensional images of horizontal cell morphology were re-constructed from 40-50 optical 1 μm sections obtained at 0.5 μm intervals. Axons were measured in micrometers from their contact with the soma to the center of the terminal arbors by tracing them on the projection image using LSM Image Analyzer. The axon collaterals or fine arbors were not included for the measurement. To calculate the area of the dendritic field, we converted the projected images to binary data and the total number of pixels was counted using Scion Image software (Scion Corporation). The image-capturing conditions such as detector gains, pinholes, laser output, and threshold for making the binary data were kept constant for quantitative analysis. The area of the dendritic field was then calculated based on the number of pixels covered by dendrites. For statistical analysis, the two-tailed Welch t-test was used.

RESULTS

Conditional expression of exogenous genes in single horizontal cells with a novel transposon-mediated gene transfer method

In order to investigate the function of cadherin during dendritic morphogenesis in vivo, we first explored an experimental method that enables conditional expression of a dominant-negative cadherin in a small set of distinct neurons at late developmental stages. We employed the chicken retina as a model system because this tissue can be easily electroporated with a foreign gene at the stage of optic vesicle (reviewed by Nakamura et al., 2004). However, the conventional method of in ovo electroporation, which is usually carried out at embryonic day 1.5 (E1.5), fails to retain the transgene into late developmental stages (reviewed by Nakamura et al., 2004) (Y.T., unpublished). We therefore used an Ac-like transposon Tol2, which originated from medaka fish Oryzias latipes (Koga et al., 1996; Kawakami, 2005) (Fig. 1A). Co-introduction of a plasmid vector expressing the Tol2 transposase (T2TP) and a plasmid that encodes a gene cassette flanked by the Tol2-cis sequence (Tol2) results in the incorporation of the latter gene cassette into the host genome, thus enabling stable gene expression in fish (Kawakami et al., 2000; Kawakami et al., 2004), ES cells (Kawakami and Noda, 2004) and chicken embryos (Y.T., unpublished). To induce exogenous gene expression in a stage-specific manner, we employed the tetracycline-mediated conditional gene expression system (Gossen et al., 1995). We thus co-electroporated the optic vesicles with three different plasmids (Fig. 1A): the first plasmid transiently expresses the Tol2 transposase; the second plasmid carries a Tol2-flanked gene cassette that expresses the mutated tetracycline repressor rtTA-M2 (Urlinger et al., 2000) under the control of the CAG promoter (Niwa et al., 1991); and the third plasmid carries a Tol2-flanked cassette that expresses membrane-targeted EGFP (mEGFP) under the control of the tetracycline responsive element (TRE, Fig. 1A) (Y.S. and Y.T., unpublished). The rtTA-M2 is a transactivator that binds the TRE sequence in the presence of the tetracycline analogue doxycycline (DOX) (Urlinger et al., 2000). No background expression was observed at any time point we observed (from E2 to E16) in the absence of DOX (data not shown). At E16, 14 days after the electroporation with these three plasmids and 24 hours after the addition of DOX, we observed that mEGFP signals are predominantly localized to a subset of cells in the retina: horizontal cells, Müller cells and a subpopulation of amacrine and retinal ganglion cells (Fig. 1B). With the method described above, only the cells that have integrated both of the Tol2-flanked gene cassettes into the genome express mEGFP upon the addition of DOX. This stochastically infrequent event leads to the advantage of a small population of cells being labeled. In addition, the horizontal cells, which we specifically focused on in this study, were positioned away from the Müller cell columns (Fig. 1B, see Fig. S1 in the supplementary material), facilitating observations of entire cell morphology at the single-cell level. The identity of the horizontal cell was confirmed by its position and morphology, as well as by the expression of molecular markers including Prox1 and Pax6 (Belecky-Adams et al., 1997; Edqvist and Hallbook, 2004; Tanabe et al., 2004) (Fig. 1B,C).
Embryonic chicken retinas have 3 morphologically distinct subtypes of horizontal cells

We next decided to use our novel method to investigate the roles of cadherins in dendritic morphogenesis. Before performing the functional analysis of cadherin, we examined the normal morphology of horizontal cells in the retina. We visualized the horizontal cells by overexpressing mEGFP, and then observed whole-mounted retina from the scleral (outer) side using confocal microscopy. As the retinal pigment epithelium (RPE) becomes tightly associated with the neural retina and hampers fluorescence observations in post-hatched chicks, we carried out our analysis at E16 and earlier, the stages during which we could still separate the RPE from the associating neural retinas. Although E16 is still an embryonic stage, synaptic ribbons and vesicles are already present in the outer plexiform layer (Hughes and LaVelle, 1974), suggesting that basic neural connecting patterns are already established.

A classic Golgi-electron microscopic (Goldi-EM) study showed that there are three subtypes of horizontal cells in the adult chicken retina (Genis-Galvez et al., 1979). We were able to identify all of these subtypes by mEGFP expression. Type I cells possessed axons and bushy dendrites that covered a small dendritic field of 230 μm² on average (n=9, s.d.=60 μm²) (Fig. 2A). The mean length of their axons was 82 μm (n=7, s.d.=9.2 μm). Type II cells were axonless, flattened cells with large, wavy dendrites covering a dendritic field of 700 μm² on average (n=7, s.d.=74 μm²) (Fig. 2B). Type III cells were also axonless, and showed a ‘candelabrum-shaped’ morphology with vertically traversing dendritic branches that were usually single up to their termination zone (Fig. 2C). The terminals of these dendrites were enlarged, forming bulge-like structures (Fig. 2C); the average dendritic area was 200 μm² (n=7, s.d.=32 μm²). To examine the ratio of each horizontal cell subtype, we examined 270 horizontal cells from 12 independent retinal regions. Among them, 93 cells (34%) belonged to the type I horizontal cells, and 68 (25%) and 109 (40%) cells were classified into type II and III horizontal cells, respectively. Our results are consistent with the aforementioned previous observations on the adult chicken retina (Genis-Galvez et al., 1979).

We then confirmed the expression pattern of N-cadherin, a major cadherin subtype expressed in the nervous system (Redies and Takeichi, 1993; Redies et al., 1993; Hatta and Takeichi, 1986), in the horizontal cells. At E16, N-cadherin mRNA was ubiquitously expressed in all the cells in the neural retina (magenta in Fig. 2E) except in the presumptive Müller cells in the inner nuclear layer (Fig. 2E). To confirm that all the horizontal cells expressed N-cadherin, we performed double labeling for Prox1 protein and N-cadherin mRNA. As expected, N-cadherin mRNA signals were observed in the cytoplasm of all the horizontal cells expressing Prox1 (Fig. 2E).

Horizontal cell dendrites undergo morphological changes during embryonic development

To determine whether the dendrites of horizontal cells undergo changes in their morphology during development, we fixed retinas at three different time points, E9, E12 and E14, and observed the morphology of the mEGFP-expressing cells (Fig. 3). At E9, EGFP-expressing horizontal cells could be found in the scleral side of the inner nuclear layer; their identity was confirmed by an intense
expression of the horizontal cell marker Prox1 (Fig. 3A). At this stage, a number of filopodia-like processes were observed projecting from the cell body (Fig. 3B), some of which projected vertically (Fig. 3B, arrowheads). All of the cells we observed possessed similar morphology, preventing us from discriminating the three subtypes of the horizontal cells. At E12, two types of horizontal cells became distinguishable. The first cell type possessed a long process reminiscent of an axon (Fig. 3C, arrow), suggesting that these cells were precursors of type I horizontal cells. Their dendrites were less branched compared with those of mature cells (Fig. 3C, Fig. 2A). Cells of the second type elongated their horizontal dendrites bi-directionally, which were randomly oriented in relation to the centroperipheral axis of the eye (Fig. 3D). These cells lacked axon-like processes, suggesting that they were precursors of type II or type III cells. The vertically protruding processes were also prominent at this stage (arrowheads in Fig. 3C,D). By E14, the vertical processes with strong mEGFP expression had disappeared, and dendritic processes had further elongated horizontally to form branching patterns characteristic of the three types of horizontal cells (Fig. 3E-G). However, the bulges of dendritic terminals of type III cells had not yet been generated; instead, growth cone-like structures were occasionally observed at the tips of the dendrites (arrows in Fig. 3G).

Fig. 4. Subtype-specific projections of dendrite terminals. (A) Serial confocal images from vitreal (left) to scleral (right) side of the outer nuclear layer. PNAL and N-cadherin expression are shown in magenta and green, respectively. The PNAL-expressing cells at the pedicle levels can be traced back to the double-cones at the level of the outer nuclear layer (asterisks). The principal- and accessory-cone pedicles are indicated by arrowheads and arrows, respectively. (B,E,H) Projection images (upper panels) and optical vertical sections (lower panels) of three types of horizontal cells double-stained with PNA and EGFP. (C,D,F,G,I,J) Higher magnification view of single optical sections taken at the level of double-cone pedicle, double-stained for EGFP and PNAL (C,F,I) or GluR4 (D,G,J). Note that the dendrite terminals of type I horizontal cells project to both the principal- and accessory-cone pedicles (arrowheads and arrows in C and I, respectively), whereas type III horizontal cells project solely to the accessory-cone pedicles (arrows in B. (K) Schematic drawing of the projection patterns of type I and type III horizontal cell dendrites. Scale bars: 5 μm in A,C,D,F,G,I,J; 10 μm in B,E,H.
Dendrite terminals project to specific synaptic sites in a subtype-specific manner

We further observed the projection patterns of the dendritic terminals of the three subtypes of horizontal cells by comparing the mEGFP signals with the ligand of peanut agglutinin (PNAL), which is expressed in cone cells in the chicken retina (Blanks and Johnson, 1984). Although PNAL was expressed in all of the cone cells at the outer segment level (Fig. 4A, right), the signals were observed in only a subset of cone cells at the pedicle level: the synaptic site of cone photoreceptor cells (Fig. 4A, left). These PNAL-positive pedicles were located at the scleral side of the outer plexiform layer, which was assumed to correspond to those of the ‘double-cone’ cells according to a previous EM study (Morris and Shorey, 1967). To confirm this interpretation, we carefully examined serial confocal sections. At the level of the outer segment, double cones were easily identified by their ‘8’ shape morphology revealed by N-cadherin immunostaining (Fig. 4A). The outlines of the double-cone cells could be traced through adjacent confocal sections, which eventually met a pair of adjacent weak and strong PNAL signals at the pedicle level (Fig. 4A, left). In all cases, the strong PNAL signals (small arrows in Fig. 4A) were surrounded by hoof-like weak PNAL signals (arrowheads in Fig. 4A). Double cones consist of two types of cone cells: principal and accessory cone cells (Morris and Shorey, 1967). Considering that the principal-cone pedicles are larger than the accessory-cone pedicles (Morris and Shorey, 1967), the weak and strong PNAL signals presumably corresponded to the pedicles of the principal and accessory cones, respectively (Fig. 4K).

The dendrite terminals of type I horizontal cells projected to the PNAL-positive sublamina of the outer plexiform layer (arrows in Fig. 4B), suggesting that they targeted the double-cone pedicles. Single optical sections at the level of the double-cone pedicles revealed that the terminals of type I horizontal cells arborized to occupy both PNAL-weak and -strong areas (arrowheads and arrows, respectively, in Fig. 4C), indicating that they projected to both principal- and accessory-cone pedicles (Fig. 4C,K). We then examined the expression of ionotropic glutamate receptor 4 (GluR4), a postsynaptic marker of horizontal cells in the chicken retina (Silveira dos Santos Bredariol and Hamassaki-Britto, 2001). The punctate signals of GluR4 were found along the dendritic terminals of type I horizontal cells, overlapping with their mEGFP signals (Fig. 4D), suggesting that these dendritic terminals formed synapses with the double-cone cells. On the other hand, the dendritic terminals of type III horizontal cells specifically projected to the portion of the double-cone pedicles with strong PNAL signals (Fig. 4I,K), which corresponded to the accessory-cone pedicles. They did not arborize but formed bulge-like structures that overlapped with the strong PNAL signals (Fig. 4I). GluR4 was co-localized with the mEGFP signals on the terminals of the type III horizontal cell.

Fig. 5. Simultaneous expression of dominant-negative cadherins and mEGFP in the horizontal cells. (A) The three vectors used for the conditional expression. The bi-directional promoter enables simultaneous expression of the dominant-negative cadherin and mEGFP marker. (B) Schematic drawing of domain structures of the dominant-negative molecules. (C-E) Expression patterns of full-length N-cadherin (C), cN390Δ (D) and cN390Δ CBR (-) (E) in mEGFP-expressing horizontal cells. Scale bar: 10 μm (C-E).
dendrites (Fig. 4J), suggesting that they also formed synapses with accessory-cone pedicles. The dendrite terminals of type II horizontal cells did not project to the PNAL-positive sublamina in the outer plexiform layer (Fig. 4E-G); instead, they terminated in the vitreal (inner) side of the outer plexiform layer, where rod spherules and single cone pedicles are predominantly distributed (Morris and Shorey, 1967). However, we could not determine the targets of the type II horizontal cells, because of a lack of molecular markers that clearly distinguish between rod spherules and single-cone pedicles at this optical resolution. Similarly, we could not clarify whether type I and type III horizontal cell dendrites also projected to other synaptic sites than the double-cone pedicles in the PNAL-negative inner region of the outer plexiform layer.

**Perturbation of cadherin function in horizontal cells decreases the dendritic field size but does not impair axon elongation**

Next, we examined whether cadherins played any role in the formation of the characteristic dendritic patterns of the horizontal cells described above. Using a plasmid vector that drives two genes simultaneously under the control of bidirectional TRE promoters, we could observe the fine morphology of electroporated cells by mEGFP expression while overexpressing another molecule in the same cell (Fig. 5A). To block the function of cadherins, we used a myc-tagged dominant-negative form of N-cadherin that lacks the extracellular domain (cN390Δ, Fig. 5B) (Fujimori and Takeichi, 1993). It has been established that cN390Δ inhibits a wide range of

*Fig. 6. cN390Δ decreases the dendritic field size of horizontal cells. (A-J) Morphology of horizontal cells expressing full-length N-cadherin (A-D) or cN390Δ (E-J) visualized by mEGFP expression at E16, 4 days after the induction of the exogenous genes. Projection images and optical vertical sections are shown in upper and lower panels, respectively. (K,L) Statistical analysis of the effect of the dominant-negative cadherin on the area of the dendritic field (K) or axonal length (L). Numbers inside the bars represent the number of cells examined. ***P<0.0001, n.s. P>0.8. Error bars represent s.d. All figures are shown at the same scale. Scale bar: 10 μm.
classic cadherin subtypes that possess a catenin-binding region (CBR) (Togashi et al., 2002; Fujimori and Takeichi, 1993). As a control, we used myc-tagged full-length N-cadherin as well as another form of the mutant molecule that lacks both the extracellular domain and the catenin-binding region (CBR), leaving the juxtamembrane domain (JM) intact [cN390Δ CBP(–), Fig. 5B]. We induced exogenous expression at E12 after the formation of the initial dendritic processes, and examined the cellular morphology after 4 days at E16. The simultaneous expression of mEGFP and the myc-tagged molecules was confirmed by double-immunostaining for mEGFP and the myc-tag on sections (Fig. 5C-E). To confirm the overexpression of cN390Δ did not cause non-specific toxic effects on cell survival, we made transverse sections that crossed the center of randomly selected mEGFP-expressing clones. The average number of labeled horizontal cells within 200 μm width retina on the sections did not change significantly by the overexpression of cN390Δ [3.6 in the control retina (n=12, s.d.=0.9) and 3.7 in the retina expressing cN390Δ (n=10, s.d.=0.8), P>0.75]. The differentiation of the three subtypes of horizontal cells occurred more or less normally, as we could distinguish the cell types by their characteristic morphology (Fig. 6E-J; see Fig. S2E-H in the supplementary material). However, the dendritic processes covered much smaller areas in the cells expressing cN390Δ than did the control ones expressing the full-length N-cadherin (Fig. 6E-J). These effects were not observed in the cells expressing cN390Δ CBP(–) (see Fig. S2E-H in the supplementary material; Fig. 6K). Statistical analysis showed that expression of cN390Δ significantly reduced the dendritic field size of all three types of horizontal cells (P<0.00008 for type I, P<0.000027 for type II and P<0.000058 for type III cells; n is shown in Fig. 6I). However, the length of axons of type I horizontal cells was not affected (Fig. 6A,E, P>0.94; n is shown in Fig. 6L). These results suggest that cadherin controls the global dendritic growth or stabilization, but not axon elongation, of horizontal cells.

To distinguish whether cN390Δ inhibited early phases of dendritic growth or de-stabilized pre-formed dendritic branches, we examined the morphology at an earlier stage, E14, 48 hours after the induction of the dominant-negative molecule (Fig. 7). At this time point, it was difficult to discriminate type II cells from type III cells by their morphology when they expressed cN390Δ (Fig. 7E-G). Therefore we compared the dendritic field size of the horizontal cells with axon (type I) or without axon (type II or type III cells). The expression of cN390Δ significantly reduced the size in both cell population (P<0.000004 for type I, P<0.001 for type II or type III cells; n is shown in Fig. 7H), suggesting that cadherin function was required during the initial phase of dendrite morphogenesis to increase the field size.

**Perturbation of cadherins did not affect local target selections but impaired synapse formation**

We then studied the projection patterns of dendritic terminals of the horizontal cells expressing cN390Δ. Interestingly, a substantial number of dendrite terminals of type I and type III horizontal cells projected to the PNAL-positive sublamina in the outer plexiform layer (arrows in Fig. 8A,C). In addition, the local target selections occurred more or less normally; i.e. the terminals of type I horizontal...
cells converged into double-cone pedicles (Fig. 8A,H) and those of type III cells projected to accessory-cone pedicles with strong PNAL signals (Fig. 8C,J). The number of dendrite terminals projecting to the correct cone pedicles from a single neuron, however, decreased upon overexpression of cN390/H9004 (P<0.000065 for type I cells and P<0.0000034 for type III cells; n is shown in Fig. 9A); however, the relative number of dendrite terminals per dendritic field area did not change significantly (P>0.08 for type I and P>0.80 for type III cells, Fig. 9B). These observations suggest that, despite the decreased size of the dendritic field, their terminals could find their correct targets within the local area they resided. The stratification of type II horizontal cells was also fairly normal, and their dendrite terminals projected to the vitreal side of the outer plexiform layer, avoiding the PNAL-positive sublamina (Fig. 8B). However, the morphologies of the dendrite terminals were severely affected; many of them covered only small areas of the double-cone and accessory-cone pedicles, respectively (arrows). GluR4 expression is either absent (arrows) or suppressed (arrowheads) in the regions that have received projections from horizontal cells expressing the cN390Δ, shown as a broken line (I,K). Scale bars: 10 μm A-C; 5 μm D-K.

In the present study, we have investigated the role of cadherin during the formation of horizontal cell dendrites at the single cell level in the developing chicken retina. Our main findings are as follows. First, we have successfully established an experimental system that enables conditional, cell-type specific expression of exogenous genes using a transposon-mediated gene transfer method. We visualized the fine morphology of horizontal cells by the expression of mEGFP and confirmed the presence of three types of horizontal cells, the dendritic terminals of which project onto specific synaptic sites. Second, comparisons of the mEGFP fluorescent signals with molecular markers revealed subtype-specific projection patterns of dendritic terminals in the double-cone pedicles. Third, we investigated the effects of dominant-negative cadherin cN390Δ on the development of horizontal cell dendrites. We found that cadherin controlled distinct steps of dendrite morphogenesis as well as synapse formation, as discussed below.

The overexpression of cN390Δ decreased the dendritic field area of the horizontal cells. During normal development, filopodia-like processes were commonly observed at earlier stages when the
dendrites were actively growing (E9-14), which disappeared at later stages such as E16. We can assume that the outgrowth of dendritic processes requires their anchoring to other cells or substrates. Considering that cadherin activity is essential for the firm attachment of filopodial processes to other neurites, as found in the case of axon-to-dendritic spine interactions (Togashi et al., 2002; Abe et al., 2004), cadherin probably sustains the extension of horizontal cell dendrites by providing them with the ability to anchor to other cells. In fact, cells expressing the dominant-negative cadherin retained a hairy morphology with a number of filopodia-like processes, which might have failed to stabilize the attachment to other cells or substrates. It remains unclear whether cadherin activity is required for the maintenance of horizontal cell dendrites. As the confocal observation of dendrite morphology was available during only a limited period of time (E16 and earlier), before the formation of tight association between the pigment epithelium and the photoreceptor cells, it was technically demanding to investigate the effect of the dominant-negative cadherin at later stages. Time lapse observation using cultured retinal explants may provide an alternative way to clarify if cadherin function is necessary for the extension of the dendritic processes or for the stabilization of the established branches. The anchorage of the dendritic processes may be mediated by the homophilic interactions of cadherins expressed by the horizontal cells with those expressed by other cells; however, we are currently ignorant of the precise cell types to which developing horizontal cell dendrites attach. As all three types of neurons comprising the outer plexiform layer express N-cadherin, the dendrites of horizontal cells potentially interact with photoreceptor cells and bipolar cells, as well as with those of the same cell type via the N-cadherin homophilic bindings. Further studies that specifically block cadherin functions in each cell type will clarify which cells horizontal cells interact during the extension of their dendrites.

Compared with the prominent defects in dendrite formation, the axon elongation of type I horizontal cells was not affected by cN390Δ, suggesting that horizontal cell axons navigate independently of cadherin activity. Interestingly, a previous study showed that overexpression of a dominant-negative cadherin inhibits outgrowth of axons and dendrites of retinal ganglion cells in the *Xenopus* retina (Riehl et al., 1996). This discrepancy might be explained by the different timing of exogenous gene expression. In *Xenopus*, the first expression of exogenous genes introduced by lipofection is observed in undifferentiated retinal progenitor cells (Riehl et al., 1996; Holt et al., 1990), whereas we induced the expression in postmitotic neurons that had finished their vertical migration across the retinal layers. Cadherin might thus be necessary for an early step of the outgrowth of axons, but dispensable for their elongation. Alternatively, the axon growth may be regulated in a cell type-specific manner, and a cadherin-dependent cell-adhesion system is differentially operating at the tips of the growth cone depending on the cell type. In fact, horizontal cell axons are unique in the sense that they do not generate neuronal impulses (Nelson et al., 1975) and hence may use different molecular machineries for elongation.

Although cN390Δ decreased the dendritic field size, local target selection of the dendrite terminals took place to some extent. For example, dendrites of type II horizontal cells correctly terminated in the vitreal side of the outer plexiform layer and never invaded the PNAL-positive sublamina located sclerally. In addition, a substantial number of dendrite terminals of type I and type III horizontal cells correctly found their targets in the double-cone pedicles, although the number of terminals decreased almost proportionally to the reduction in the dendritic field of each cell. We, thus, speculate that cadherins, at least the subtypes that can be inhibited by the dominant-negative construct used here, are not required for the local target selection of dendrite terminals in horizontal cells but that they control global extension or stabilization of the dendrites as discussed above.

Postsynaptic marker accumulation was largely impaired in the cells expressing cN390Δ. This observation is consistent with a series of preceding in vitro studies suggesting the essential role of cadherins in synapse formation (reviewed by Shapiro and Colman, 1999; Uemura, 1998; Takeichi and Abe, 2005). The defective synaptic contacts may partially be responsible for the suppressed extension of dendrites. For example, a reduction in the mechanical forces required to maintain the synaptic contacts may allow a contraction of dendrites, leading to smaller dendritic fields. Furthermore, normal synaptic activities might be essential for maintaining the normal morphology of dendrites, as found in the case of other neurons (reviewed by Wong and Ghosh, 2002). The defective synaptic contacts may allow a contraction of dendrites, leading to smaller dendritic fields.

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specifications of various promoters (Sive et al., 2000), making it difficult to perform cell type-specific expression, let alone stage-specific conditional expression. As for retroviruses, making high-titer virus is time-consuming and even impossible, especially when the insert length exceeds the capacity of the virus vector. The transposon-mediated gene transfer system we have used in this study provides a cell type-specific, inducible overexpression system with conventional plasmid expression vectors. The use of different tissue-specific promoters enabled the induction of exogenous genes in distinct cell types (K.T. and S.N., unpublished), providing further opportunity to examine the function of specific genes in the nervous system. We believe the experimental method used in this study offers a conventional and alternative experimental approach to the genetic method (Zong et al., 2005) for analyzing gene function at the single-cell level in the vertebrate nervous system.

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Supplementary material

Supplementary material for this article is available at Supplementary material online.

References


