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NinaB Is Essential for *Drosophila* Vision but Induces Retinal Degeneration in Opsin-deficient Photoreceptors*[S]

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In animals, visual pigments are essential for photoreceptor function and survival. These G-protein-coupled receptors consist of a protein moiety (opsin) and a covalently bound 11-*cis*-retinylidene chromophore. The chromophore is derived from dietary carotenoids by oxidative cleavage and *trans*-to-*cis* isomerization of double bonds. In vertebrates, the necessary chemical transformations are catalyzed by two distinct but structurally related enzymes, the carotenoid oxygenase β-carotenoid-15,15′-monooxygenase and the retinoid isomerase RPE65 (retinal pigment epithelium protein of 65 kDa). Recently, we provided biochemical evidence that these reactions in insects are catalyzed by a single enzyme family member named NinaB. Here we show that in the fly pathway, carotenoids are mandatory precursors of the chromophore. After chromophore formation, the retinoid-binding protein Pinta acts downstream of NinaB and is required to supply photoreceptors with chromophore. Like *ninaE* encoding the opsin, *ninaB* expression is eye-dependent and is activated as a downstream target of the *eyeless/pax6* and *sine oculis* master control genes for eye development. The requirement for coordinated synthesis of chromophore and opsin is evidenced by analysis of *ninaE* mutants. Retinal degeneration in opsin-deficient photoreceptors is caused by the chromophore and can be prevented by restricting its supply as seen in an opsin and chromophore-deficient double mutant. Thus, our study identifies NinaB as a key component for visual pigment production and provides evidence that chromophore in opsin-deficient photoreceptors can elicit retinal degeneration.

Photoreceptors are sensory neurons with specialized light-sensitive compartments, the outer segments of vertebrate cones and rods and the rhabdomeres of insects. These structures are orderly stacks of photosensitive plasma membrane that house the phototransduction machinery. Light sensitivity is mediated by visual pigments, the major protein constituent of these membranes. Visual pigments are G-protein-coupled receptors that consist of an integral membrane protein, referred as the opsin, and an 11-*cis*-retinylidene chromophore covalently linked to a lysine residue through a Schiff base linkage (reviewed in Ref. 1). Visual pigment biogenesis is a complex process that involves the transport of the opsin through the secretory pathway to photosensitive membranes and the metabolic supply of the chromophore. It is now clear that mutations resulting in ineffective visual pigment biogenesis and pigment regeneration after a bleach are an important cause of retinal degeneration and blindness in humans (for recent reviews see Refs. 2, 3).

Drosophila have long served as an animal model to analyze visual pigment biogenesis and study the pathology of associated blinding diseases (for recent review see Ref. 4). The first mutations linked to retinal degeneration were noted in the *ninaE* gene (neither inactivation nor afterpotential mutant E) (5, 6). *ninaE* encodes the opsin moiety of the flies' major visual pigment (Rh1)[2] and is expressed in photoreceptors R1–R6 (7, 8). In *ninaE* null mutants, rhabdomeres degenerate and their membranes involute into the cytoplasm (9). This phenotype develops light independently and does not require downstream factors of the G-protein signaling cascade (4). Therefore, an essential structural role for opsin in building rhabomeric membranes during photoreceptor morphogenesis has been proposed (9, 10). In addition to opsin, the chromophore is also crucial for visual pigment biogenesis. In flies, defects in chromophore production prevent normal expression and stability of the opsin (11, 12). In chromophore deficiency, the opsin virtually disappears from photoreceptor cells (12). However, the converse situation to examine the consequences of chromophore deficiency for photoreceptor morphogenesis has not been studied in great detail in the fly.

The chromophore (11-*cis*-retinal in vertebrates and 11-*cis*-3-hydroxy-retinal in flies) is a retinoid (C20) derived from carotenoids (C40) by oxidative cleavage and *trans*-to-*cis* isomerization of double bonds. Analysis of blind *Drosophila* mutants has identified several genes required for synthesis of the chromophore (13–16). Among these mutants, *ninaB* flies cannot convert carotenoids to retinoids (14, 16). The *ninaB* gene

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[2] The abbreviations used are: Rh1, visual pigment of rhabdomeres R1–R6; BCMO1, β-carotenoid-15,15′-monooxygenase; NinaB, neither inactivation nor afterpotential mutant B; RPE65, retinal pigment epithelium protein of 65 kDa; Pinta, prolonged depolarization afterpotential is not apparent mutant; STRA6, stimulated by retinoic acid gene *6*; RBP4, serum retinol-binding protein; BO, Bolwig's organ; *so*, *sine oculis*; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HPLC, high-performance liquid chromatography.

encodes a protein that belongs to a family of structurally related non-heme iron oxygenases (17) that include the mammalian carotenoid-oxygenase BCMO1 and the retinoid isomerase RPE65 (retinal pigment epithelium protein of 65 kDa) (17). These enzymes catalyze two key steps in the pathway for chromophore production, the conversion of carotenoids to retinoids by oxidative cleavage (18) and the *trans*-to-*cis* isomerization of the C10,C11 double bond of retinoids (19–22). Dietary supplementation with preformed retinoids can compensate for BCMO1 deficiency (19), but RPE65 is essential for chromophore production and regeneration (23). In humans, mutations in RPE65 cause Leber congenital amaurosis or the less severe, later onset retinitis pigmentosa (reviewed in Ref. 3).

In flies, an 11-*cis* configuration of retinoids is also essential for visual pigment biogenesis (12, 25, 26). Although preformed retinoids such as all-*trans*-retinal support visual pigment production only in the presence of light, carotenoids can promote visual pigment production light independently (25). Biochemical analysis shows that NinaB from the moth *Galleria mellonella* combines the activities of a carotenoid oxygenase and a retinoid isomerase in a single polypeptide (25). In this isomerooxygenase reaction, carotenoids such as zeaxanthin are light-independently converted to one molecule of 11-*cis*- and one molecule of all-*trans*-3-hydroxy-retinal. This finding suggests that NinaB and carotenoids are essential for visual pigment biogenesis and photoreceptor morphogenesis. Therefore, we here analyzed the role of NinaB in this process in *Drosophila* photoreceptors that are amenable for genetic, biochemical, and structural analyses.

EXPERIMENTAL PROCEDURES

Drosophila Maintenance, Fly Strains, and Supplementation Experiments—Flies were reared on standard corn medium at 25 °C in a 16-h light/8-h dark environment unless noted otherwise. Strains were *ninaB*^{360d}, Oregon R, wild-type Berlin, *ninaD*^{P245}, *ninaE*^{17ol}, *sine oculis*, and *yellow white*. To obtain flies with ectopic eyes, Bloomington fly strains #1553 and #6294 were crossed (27). To generate flies ectopically expressing the UAS-*ninaB*$^{(wt)}$ transgene, UAS-*ninaB*$^{(wt)}$/UAS-*ninaB*$^{(wt)}$; TM2/MKRS flies were crossed with +/CyO; *tubulin*-GAL4/TM3 flies. *ninaD*^{P245}; *ninaE*^{17ol} double mutant flies were obtained by appropriate crossings to yield a fertile strain with the genotype *ninaD*^{P245}/CyO; *ninaE*^{17ol}/*ninaE*^{17ol}. From this strain, *ninaD*^{P245}/*ninaD*^{P245}; *ninaE*^{17ol}/*ninaE*^{17ol} flies were selected for experiments.

For supplementation experiments, flies were raised on carotenoid-depleted media containing the tested supplement, *e.g.* 500 μM all-*trans*-retinal, 5 μM β-carotene, or 5 μM zeaxanthin. Early pupae were then transferred to fresh vials containing carotenoid-depleted media. Two days after eclosion, adult flies were used for Western blot analysis for Rh1. For investigation of the role of all-*trans*-retinal in retinal degeneration, freshly eclosed *ninaD*^{P245}; *ninaE*^{17ol} flies were transferred to media supplemented with ethanol alone (control) or ethanol plus all-*trans*-retinal (500 μM) for 2 days. Carotenoids were purchased from Wild (Heidelberg, Germany); all-*trans*-retinal was purchased from Sigma.

Whole Mount in Situ Hybridization of Drosophila Embryos—Embryo dechorionation and *in situ* hybridization were performed as described (14). The *ninaB* probe was synthesized by using the primers up, 5′-CTAAATGGCATTGGGTGCAAACC-3′, and down, 5′-ACCTGGGCACCACAATGA-3′.

Light Avoidance Assay—Foraging third instar larvae of *ninaB*^{360d} and Oregon R strains were tested in a light *versus* dark preference test at 750 lux as described (28). Experiments were conducted in triplicate using 20 larvae for each test.

Estimation of mRNA Levels by Semiquantitative RT-PCR Analyses—RT-PCR analyses for *ninaB* were carried out as previously described (29).

Extraction of Carotenoids and Retinoids and HPLC Analyses—Lipid compounds from flies were extracted and HPLC procedures were carried out as described previously (17, 25, 29). All extractions were carried out under a red safety light.

Western Blot Analyses—Western blot analyses of proteins obtained from fly heads were carried out as previously described (29).

Assessment of Retinal Toxicity—*Drosophila* S2 cells were cultured in Schneider's *Drosophila* medium (Invitrogen) as previously described (29). Cells were suspended in medium for 24 h at 24.5 °C containing either 1% DMSO alone, or either all-*trans*-retinol or all-*trans*-retinal, each dissolved in DMSO. Cell viability was estimated by measuring the percentage of cells that failed to stain with Trypan Blue (0.4%, Sigma-Aldrich) after exposure according to the manufacturer's protocol. Cells were then loaded into a hemocytometer chamber, and numbers of non-viable (stained) and viable (non-stained) cells were counted under a microscope.

Test for Enzymatic Activity—For heterologous expression, expression vectors for *Drosophila* and *Galleria* NinaB (25), were transformed in the *Escherichia coli* strain XL1-blue (Stratagene, La Jolla, CA). Bacteria were grown at 28 °C to an A_{600} of 0.5. Protein expression was induced with L-arabinose (0.02% w/v) for 8 h. Bacteria were harvested by centrifugation and broken with a French press in a buffer containing 50 mM Tricine/KOH (pH 7.6, 4 °C), 100 mM NaCl, and protease inhibitor mixture (Roche Molecular Diagnostics, Pleasanton, CA). The crude protein extract was centrifuged at 20,000 × *g* for 10 min, 4 °C. Reactions were run in 2-ml Eppendorf tubes in a volume of 100 μl in 50 mM Tricine/KOH (pH 7.6) with 3% (w/v) 1-*S*-octyl-β-D-thiopyranoside in the presence of 20 μM zeaxanthin. Retinoids and carotenoids were extracted as previously described (25). Samples were dissolved in methanol/acetonitrile (50:50, v/v) and subjected to HPLC separation on a C18 column (Zorbax Eclipse XDB, 4.6 × 1500 m, 5 μm, Agilent Technologies, Santa Clara, CA) followed by mass spectrometry. Detection of 3-hydroxy-retinaloxime was acquired using an LXQ high throughput linear ion trap mass spectrometer (Thermo Scientific, Waltham, MA) interfaced with an atmospheric pressure chemical ionization source and series 1100 HPLC system (Agilent Technologies) consisting of a vacuum degasser, a binary pump, an autosampler with cooled sample tray, thermostatically controlled column compartment, and diode array detector.

Electron Microscopy and Immunogold Labeling—For ultrastructural analyses, *Drosophila* heads were fixed in 2.5% glutar-

aldehyde in cacodylate buffer, washed with cacodylate buffer, postfixed in 1% osmium tetroxide, and incubated in 1% uranyl acetate. For immunogold labeling, fly heads were fixed in 4% paraformaldehyde, 0.2% glutaraldehyde. Postfixation was done with 8% formaldehyde. All samples were dehydrated by an ethanol series and embedded in Epon. Sections for immunogold labeling were probed with a polyclonal anti-Rh1 rabbit antibody and a secondary anti-rabbit antibody conjugated with gold particles. Microscopy was accomplished with a Philips CM10 electron microscope.

F-actin Staining—Fly heads were fixed in 2% paraformaldehyde for 1.5 h and incubated with increasing sucrose concentrations up to 50% overnight. Then 10-μm cryosections were cut, transferred to poly-L-lysine-coated coverslips, and postfixed in 2% paraformaldehyde. Blocking was done in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% saponin for 2 h. Sections were incubated in phalloidin-rhodamine overnight at 4 °C. Samples were mounted and viewed by confocal microscopy (Leica TCS-SP).

RESULTS

NinaB Is Essential for Larval Vision—In *Drosophila*, the first light-sensitive structure develops during embryogenesis. The larval Bolwig's organ (BO) entrains the clock of pacemaker neurons and also mediates larval photophobic behavior. The BO is composed of 12 photoreceptors, 8 expressing Rh6 and 4 expressing Rh5 (30). It is not clear how the BO acquires chromophore for the production of these visual pigments. To analyze the role of NinaB in this process, we performed whole mount *in situ* hybridization with staged embryos. Staining for *ninaB* mRNA was found at embryonic stage 13 and persisted until stage 16 (Fig. 1*A*). At stage 13, staining was localized at the outermost posterior end of the head capsule, directly at the epidermis and in the immediate vicinity of the developing optic lobes. At later developmental stages, the stained cells lost contact to the outer surface of the epidermis and migrated anteriorly toward the midline. At stage 16, stained cells were located on both sides of the pharynx. This spatiotemporal expression pattern of *ninaB* mirrors the organogenesis of the BO (31). To provide evidence for a role of NinaB in BO function, we analyzed the consequences of NinaB deficiency for larval vision. Larvae are photophobic, and their behavior can be tested by a previously described assay (28). On a test plate that is half illuminated and half dark, wild-type larvae migrated preferentially to the dark side of the test plate. In contrast, *ninaB* mutant larvae were randomly distributed between the illuminated and dark areas (Fig. 1*B*). Thus, *ninaB* mutants showed no photophobic behavior indicating that NinaB is essential for larval light perception.

NinaB Expression Depends on Presence of the Eyes—Later in the life cycle, the *Drosophila* compound eye develops from the eye imaginal disk. This process is completed with the establishment of visual pigments. *ninaB* is expressed at late pupal stages coincident with the expression of opsin (29). Based on a genome-wide microarray analysis in *sine oculis* (*so*) flies that lack compound eyes, *ninaB* mRNA is ~150-fold enriched in normal eyes (32). RT-PCR analysis confirmed that *ninaB* mRNA levels were highly reduced in *so* flies (Fig. 2*A*). Some

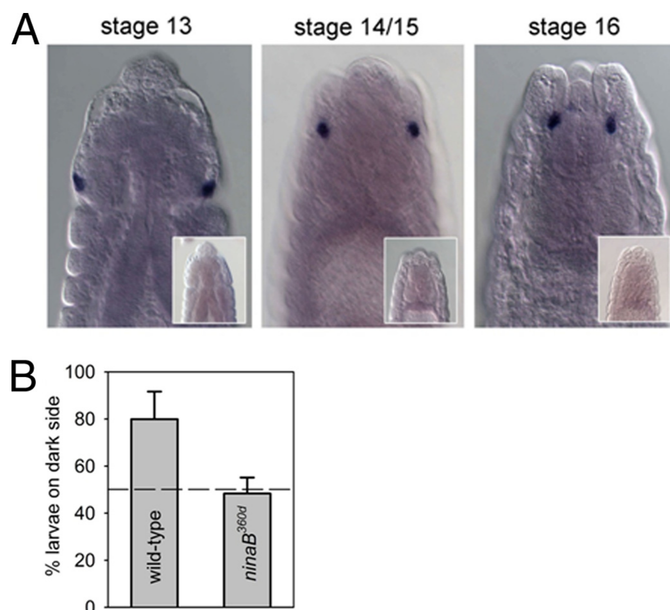

FIGURE 1. **NinaB expression and function in larval vision.** *A*, *in situ* hybridization for *ninaB* mRNA expression in embryonic stage 13–16 larvae. In stage 13, staining was localized at the outermost posterior end of the head capsule, directly at the epidermis and in the immediate vicinity of the developing optic lobes. At later developmental stages, the stained cells lost contact with the outer surface of the epidermis. In stage 16, stained cells were located on both sides of the pharynx. *Insets* show no staining with a sense *ninaB* mRNA probe. *B*, light avoidance assay performed with foraging third instar larvae of *ninaB*^{360d} mutants and wild-type flies. The *graph* exhibits the average percentages of larvae in the dark area after 15 min. Values represent three independent experiments with 20 larvae each. *Error bars* indicate the standard deviation.

residual *ninaB* mRNA expression in *so* flies is likely attributable to the eyelets that develop from the larval BO and prevail in this mutant (33). In accordance with highly reduced *ninaB* expression, HPLC analysis showed that *so* flies lacked the chromophore but accumulated carotenoid precursors (Fig. 2*C*). Thus, *ninaB* expression and chromophore production depend on the presence of compound eyes. To provide additional evidence that expression of *ninaB* and chromophore production is dependent on eye formation, we used a fly strain (*dpp*-Gal4≫UAS-*eyeless*) that develops ectopic eyes. These structures have the characteristics of compound eyes, including groups of fully differentiated ommatidia with a complete set of photoreceptor cells that are light-sensitive (27). RT-PCR analysis revealed that *ninaB* mRNA expression was induced in thoraces of pupae with ectopic eyes but was not detectable in thoraces of wild-type flies (Fig. 2*B*). Thus, *ninaB* expression and chromophore production is governed by *eyeless* and *so*, major control genes for eye development.

Carotenoids and NinaB Are Essential for Rh1 Production during Compound Eye Development—In *Drosophila*, retinoid production was eye-dependent (see above), and biochemical studies showed that NinaB combines the activities of vertebrate BCMO1 and RPE65 (25). Therefore, we asked whether carotenoids are mandatory for visual pigment biogenesis in *Drosophila*. To answer this question, we supplemented larvae with either carotenoids or preformed retinoids. The resulting pupae were then transferred to carotenoid- and retinoid-free medium and kept under a 16-h/8-h light/dark cycle. We then measured

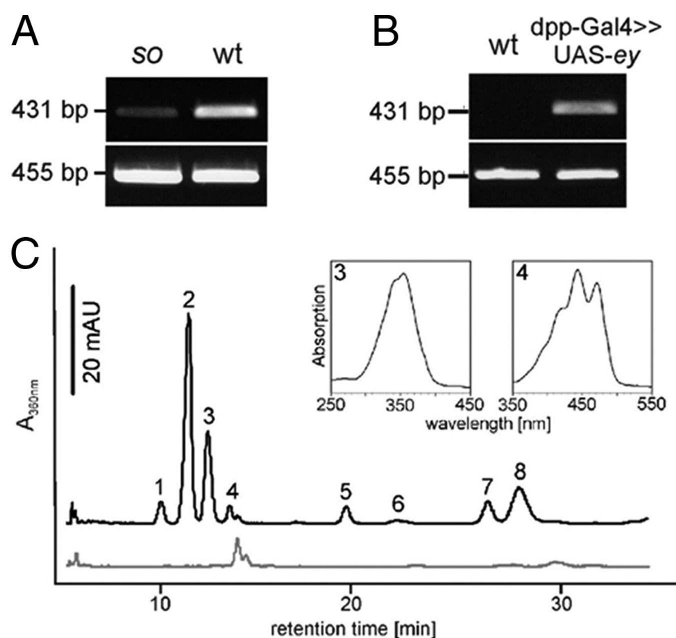

FIGURE 2. **NinaB expression and chromophore production is eye-dependent.** *A*, semiquantitative RT-PCR analysis for *ninaB* expression with total RNA preparations from the heads of adult *sine oculis* and wild-type flies. *B*, semiquantitative RT-PCR analysis for *ninaB* mRNA expression with total RNA preparations isolated from thoraces of flies with ectopic eyes induced by *eyeless/pax6* expression (dpp-GAL4≫UAS-*ey*) and thoraces of wild-type flies. Analysis for *rp49* (ribosomal protein 49) mRNA expression was used as the control. *C*, HPLC traces obtained from lipid extracts of 70 mg of wild-type (*upper trace*) and *sine oculis* (*lower trace*) fly heads. Different retinoids and carotenoids are indicated by *numbers*. *Peaks*: *1*, 9/13-*cis*-3-hydroxy-retinal oxime (*syn*); *2*, all-*trans*- (*anti*) and 11-*cis*-3-hydroxy-retinal oxime (*syn*); *3*, all-*trans*-3-hydroxy-retinal oxime (*syn*); *4*, zeaxanthin/lutein; *5*, all-*trans*-3-hydroxy-retinol; *6*, 9-*cis anti*-3-hydroxy-retinal oxime; *7*, 13-*cis*-3-hydroxy-retinol; and *8*, 11-*cis*-(*anti*)-3-hydroxy-retinal oxime. *Insets* show the spectra of *peak 3*, all-*trans*-retinal oxime (*syn*) and *peak 4*, zeaxanthin.

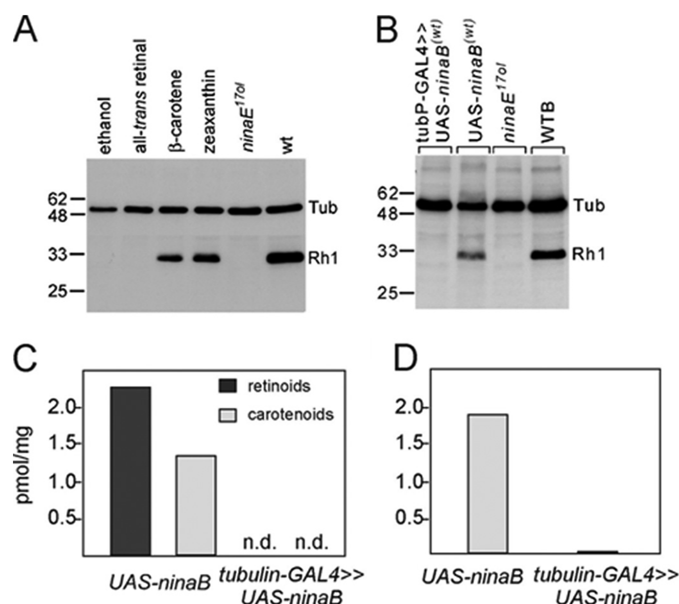

FIGURE 3. **Carotenoids are essential precursors for visual pigment production.** *A*, Western blot analysis of Rh1 production with total protein extracts from adult fly heads. Flies were raised from larva supplemented with different carotenoid and retinoid precursors for chromophore production. $ninaE^{17ol}$ (*rh1* null), and wild-type flies reared on standard corn medium were used as controls. Tubulin was used as the loading control. *B*, Western blot analysis for Rh1 with protein extracts from heads of the *tubulin*-GAL4≫UAS-*ninaB*$^{(wt)}$ and UAS-*ninaB*$^{(wt)}$ flies. Tubulin was used as loading control. *C*, carotenoid and retinoid content of the heads of *tubulin*-GAL4≫UAS-*ninaB*$^{(wt)}$ and UAS-*ninaB*$^{(wt)}$ flies. *D*, carotenoid content of the trunks of *tubulin*-GAL4≫UAS-*ninaB*$^{(wt)}$ and UAS-*ninaB*$^{(wt)}$ flies. *n.d.*, not detectable.

Rh1 levels in freshly eclosed adult flies by immunoblot analysis. Mature Rh1 has a molecular mass of 32 kDa, indicative of post-translational processing and translocation to rhabdomere membranes (12). In this experimental setup, carotenoid-supplemented larvae produced Rh1 (Fig. 3*A*). In contrast, all-*trans*-retinal supplementation did not support Rh1 production (Fig. 3*A*), indicating that *Drosophila* lacks a transport system for dietary retinoids to support visual pigment biogenesis.

To provide further evidence for the hypothesis that flies lack a transport system for dietary retinoids, we analyzed the consequences of ectopic *ninaB* expression by using a UAS/GAL4 expression system (34). We established a fly strain that expressed a UAS-*ninaB* wild-type transgene under the control of a *tubulin*-GAL4 driver in the whole fly. HPLC analysis revealed that the carotenoid and retinoid contents of these flies were dramatically reduced (Fig. 3, *C* and *D*). Immunoblot analysis showed that these flies also lacked Rh1 (Fig. 3*B*). This finding suggests that chromophore production outside the eyes reduced carotenoids available for chromophore production in the eyes, thereby impairing visual pigment production.

Zeaxanthin Is the Precursor for Chromophore Production—Flies use 3-hydroxy-retinal as chromophore. We found that both non-hydroxylated and hydroxylated carotenoids supported visual pigment production during compound eye development. Previously, it has been shown that β-carotene is converted via cryptoxanthin into zeaxanthin when fed to larvae, indicating that hydroxylated carotenoids are the direct precursor for chromophore (35). However, tests for enzymatic activity with zeaxanthin and recombinant NinaB failed in a previous study (17). We therefore applied the modified test conditions that were recently established for *Galleria* NinaB (25). We confirmed that recombinant *Galleria* NinaB efficiently converts zeaxanthin to 3-hydroxy-retinal (Fig. 4, *A* and *B*). Recombinant *Drosophila* NinaB proved to be less active, but mass spectrometry revealed that 3-hydroxy-retinal was produced (Fig. 4*C*). Thus, β-carotene is first converted to zeaxanthin and then cleaved by NinaB to directly yield 3-hydroxy-retinal.

Both Retinoid Cleavage Products of a Carotenoid Are Converted to the Chromophore—Biochemical analysis showed that NinaB converts one half-site of a carotenoid to 11-*cis*-retinal, whereas the other half-site remains in the all-*trans* configuration (25). Accordingly, zeaxanthin supplementation resulted in light-independent Rh1 production (Fig. 5*A*), and HPLC analysis revealed that 11-*cis*-3-hydroxy-retinal and all-*trans*-3-hydroxy-retinal existed in equal molar amounts in flies (Fig. 5*B*). In illuminated flies, this ratio was altered in favor of the 11-*cis*-3-hydroxy-retinal stereoisomer (Fig. 5*B*). This increase of 11-*cis*-3-hydroxy-retinal indicated that the all-*trans*-retinal cleavage product is converted in a light-dependent manner to the chromophore. A light-dependent pathway for chromophore production from all-*trans*-retinal has been described for adult flies (12). However, chromophore production from all-*trans*-retinal relies on an unspecific diffusion rather than on a protein-facilitated transport process. When we applied a solu-

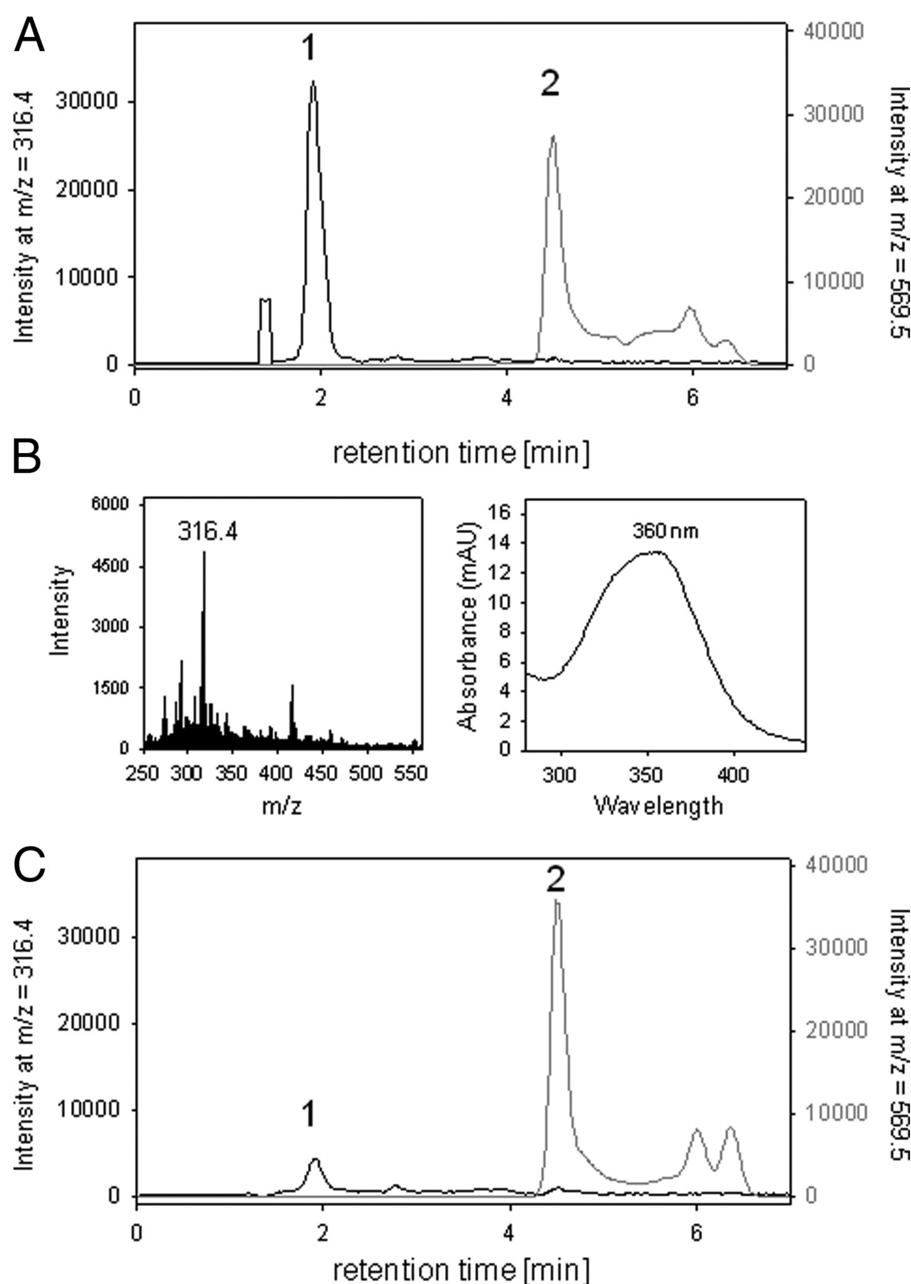

FIGURE 4. **NinaB converts zeaxanthin into 3-hydroxy-retinoids.** *A*, HPLC profile of an enzymatic test with recombinant *Galleria* NinaB. Single extracted ion chromatograms for 3-hydroxy-retinal oxime (*m/z* = 316.4; *peak 1*) and zeaxanthin (*m/z* = 569.5; *peak 2*) are shown. *B*, molecular identity of zeaxanthin cleavage product was confirmed based on the mass spectrum (*left panel*) and UV-visible absorbance spectrum (*right panel*) recorded between 1.8 and 2.2 min of elution. Molecular mass of 316.4 and maxima of absorbance spectrum around 360 nm correspond to 3-hydroxy-retinal oxime. *C*, HPLC profile of an enzymatic test with recombinant *Drosophila* NinaB. Intensities at *m/z* 316.4 (*peak 1*, 3-hyydroxy-retinal oxime) and at *m/z* 569.5 (*peak 2*, zeaxanthin) is shown.

tion of all-*trans*-retinal directly on the eyes of adult *ninaB* mutants, Rh1 production was restored in a light-dependent manner (Fig. 5*C*). Thus, we conclude that light-dependent *trans*-to-*cis* isomerization of retinoids is required for the conversion of the all-*trans*-retinal cleavage product of carotenoids. This light-dependent pathway ensures that both retinoid cleavage products can be utilized for chromophore synthesis.

The Retinoid-binding Protein Pinta Is Required to Supply Photoreceptors with Chromophore—Our analysis showed that *Drosophila* apparently lack a transport system for retinoids. However, a retinoid-binding protein, designated Pinta, was identified that is expressed in pigment cells of the eyes (36). Pinta is a homolog of the vertebrate cellular retinal binding protein CRALBP that acts downstream of retinoid isomerase RPE65 (37). Pinta mutants lack Rh1, but the biochemical consequences of the *pinta* null mutation for chromophore production have not been characterized. Therefore, we determined the retinoid composition in the heads of *pinta* flies by HPLC analysis. Both 11-*cis* and all-*trans*-3-hydroxy-retinal were detectable in *pinta* flies (Fig. 6*B*). Additionally, 3-hydroxy-retinols existed in amounts comparable to wild-type flies (Fig. 6*B*). Immunoblot analysis revealed that, despite production of chromophore, Rh1 levels were highly reduced in this mutant (Fig. 6*A*). This finding indicates that Pinta is not required for chromophore production. Lack of Rh1 in this mutant rather suggests that Pinta acts downstream of NinaB and facilitates the transport of chromophore from its locus of NinaB-dependent production to photoreceptor cells.

Rhabdomeres of the ninaB Mutant Do Not Degenerate—Our studies indicated that visual pigment production essentially depends on NinaB. Indeed, Rh1 was absent in *ninaB* mutants (Fig. 5*C*). Because photoreceptors degenerate in mutants that that lack opsin, it has been suggested that Rh1 is of critical importance for photoreceptor morphogenesis (9). Therefore, we asked whether photoreceptors of *ninaB* mutant flies also underwent retinal degeneration. Surprisingly, the overall structure of photoreceptors in chromophore-deficient *ninaB* mutants was intact (Fig. 7, *A* and *B*). Although some photoreceptors developed two rhabdomeres instead of one, this abnormality is clearly distinguishable from retinal degeneration in opsin-deficient mutants, characterized by disintegration of rhabdomeric membranes (9). To exclude the possibility that opsin was translocated to rhabdomeres in the *ninaB* null mutant and prevented retinal degeneration, we performed immunostaining for Rh1. No evidence for the presence of opsin at rhabdomeres of *ninaB* mutants was found (Fig. 7*D*), whereas opsin was readily detectable in wild-type flies (Fig. 7*C*). Thus, although *ninaB* null

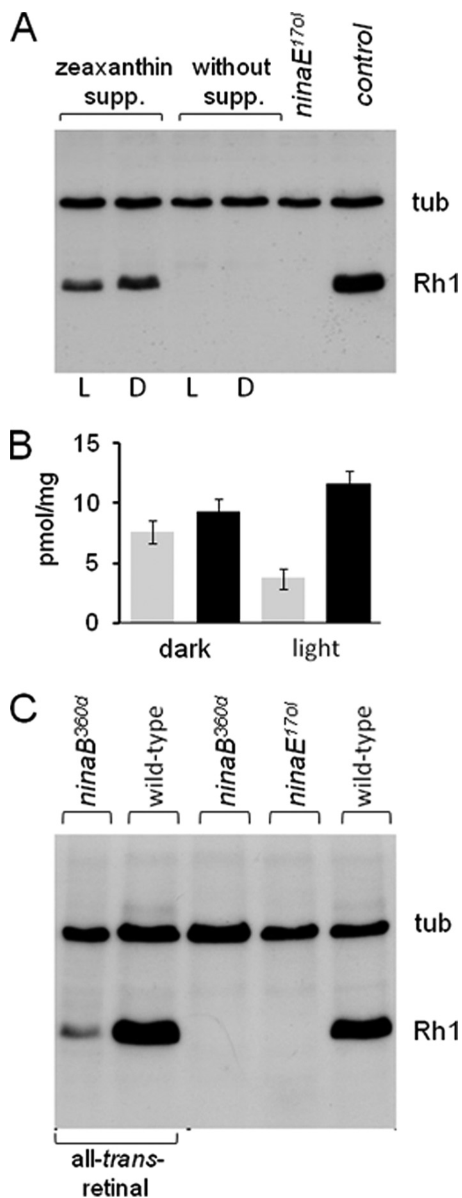

FIGURE 5. **The all-*trans*-retinal cleavage product of carotenoids is light-dependently converted to chromophore.** *A*, Western blot analysis for Rh1 in zeaxanthin-supplemented and nonsupplemented wild-type flies. After supplementation, flies were kept either in a light/dark cycle, *L*, or in darkness, *D*. *ninaE^{17ol}* (*rh1* null) and wild-type flies reared on standard corn medium were used as controls. *B*, composition of 11-*cis*-3-hydroxy-retinal (*black bars*) and all-*trans*-3-hydroxy-retinal (*gray bars*) in wild-type flies reared in the dark and in a light/dark cycle. The values give the mean ± S.D. ($n = 3$ per light condition). *C*, application of all-*trans*-retinal onto the eyes rescues defects in Rh1 production of the *ninaB* mutant. Western blot analysis for Rh1 with total protein extracts of fly heads after application of all-*trans*-retinal directly onto the eyes. All-*trans*-retinal was dissolved in ethanol (100 μM) and applied directly to the compound eyes using a fine brush. Rh1 levels were determined 2 days after application of all-*trans*-retinal. *ninaE^{17ol}* (*rh1* null) and wild-type flies reared on standard corn medium were used as controls. Tubulin was used as the loading control.

mutants lack Rh1 at rhabdomeres, these membranes did not degenerate as in opsin-deficient mutants.

Chromophore Deficiency Can Prevent Retinal Degeneration in Opsin Null Mutants—Visual pigment deficiency caused by mutations in either *ninaB* or *ninaE* genes have dramatically different consequences for photoreceptors. Because the chromophore is present in *ninaE* mutants (supplemental Fig. S1), whereas in the *ninaB* mutants it is not (25), we speculated that retinal degeneration in the opsin deficiency is caused by the chromophore. To provide evidence for this hypothesis, we attempted to suppress retinal degeneration by chromophore deficiency. Because the *ninaE* and *ninaB* genes are located close together on the same chromosome, we took advantage of the *ninaD* mutant. *ninaD* null mutant flies cannot absorb dietary carotenoids and thus lack chromophore (14). We generated an opsin- and chromophore-deficient *ninaE*;*ninaD* double mutant by appropriate crossings. We then compared photoreceptor morphology in *ninaE* single, *ninaE*;*ninaD* double mutants, and wild-type flies by F-actin staining of rhabdomeres. Confocal imaging revealed that rhabdomeres in the double mutant were arranged in regular patterns as in wild-type photoreceptors (Fig. 8, *A*, *B*, *E*, and *F*). In contrast, photoreceptors of the *ninaE* single mutant exhibited largely disorganized rhabdomeres (Fig. 8, *C* and *D*).

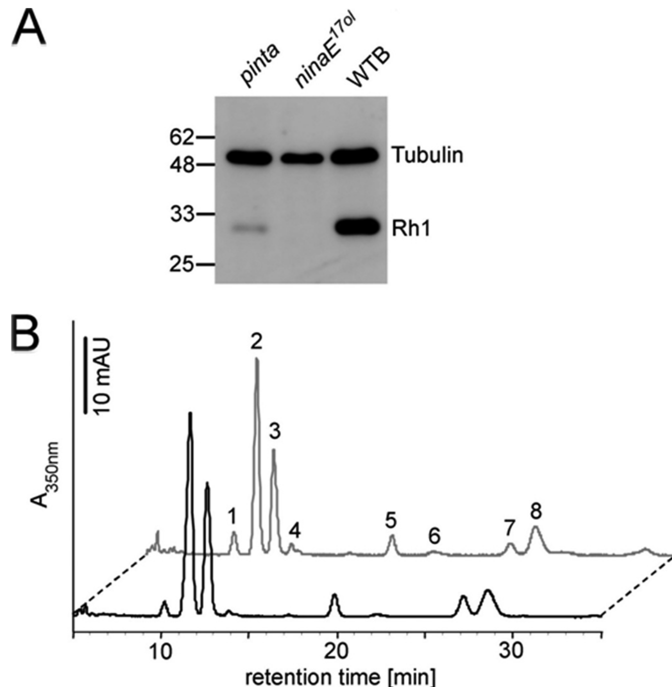

FIGURE 6. **Pinta is required for delivery of chromophore to photoreceptors.** *A*, Western blot analysis for Rh1 with total protein extracts of *pinta* fly heads. *ninaE^{17ol}* (*rh1* null) and wild-type flies reared on standard corn medium were used as controls. Tubulin was used as the loading control. *B*, HPLC profile at 350 nm of lipophilic extracts from 50 mg of wild-type (*lower trace*) and *pinta* fly heads (*upper trace*). Flies were reared on standard corn medium containing only carotenoids. Different retinoids and carotenoids are indicated by *numbers*. *Peaks*: *1*, 9/13-*cis*-3-hydroxy-retinal oxime (*syn*); *2*, all-*trans*- (*anti*) and 11-*cis*-3-hydroxy-retinal oxime (*syn*); *3*, all-*trans*-3-hydroxy-retinal oxime (*syn*); *4*, zeaxanthin/lutein; *5*, all-*trans* 3-hydroxy-retinol; *6*, 9-*cis*-(*anti*)-3-hydroxy-retinal oxime; *7*, 13-*cis*-3-hydroxy-retinol; and *8*, 11-*cis*-(*anti*)-3-hydroxy-retinal oxime.

Retinal Can Provoke Cell Damage—Our results with the *ninaE*;*ninaD* double mutant showed that lack of chromophore can suppress the degeneration phenotype of the *ninaE* null mutant. Conversely, this suggests that the presence of chromophore is required for retinal degeneration in the opsin-deficient photoreceptors. To provide evidence that the chromophore is toxic to cells, we treated *Drosophila* Schneider S2 cells with all-*trans*-retinal. Trypan blue staining of cells revealed that all-*trans*-retinal caused cell death in a dose-de-

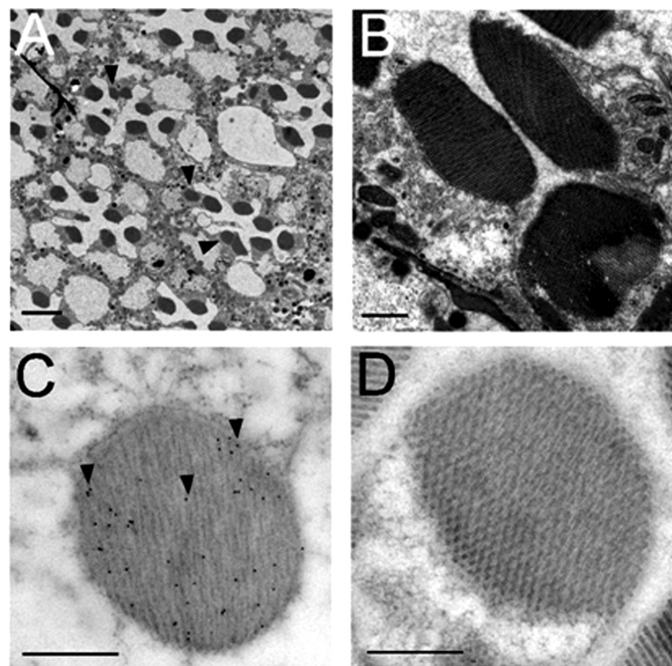

FIGURE 7. **Photoreceptors of *ninaB* null mutant flies are structurally intact.** *A* and *B*, electron micrographs of cross-sections through the compound eyes of a 3 days post eclosion *ninaB*^{360d} mutant at low (*A*) and high (*B*) magnification. *Arrowheads* indicate photoreceptor cells, which formed two rhabdomeres. *Scale bar* in *A*, 5 μm and in *B*, 1 μm. *C* and *D*, Immunogold labeling for Rh1 in cross-sections of rhabdomeres from 3 days post eclosion wild-type (*C*) and *ninaB*^{360d} mutant (*D*) flies. *Arrowheads* indicate immunogold staining for Rh1. *Scale bars* in *C* and *D*, 0.5 μm.

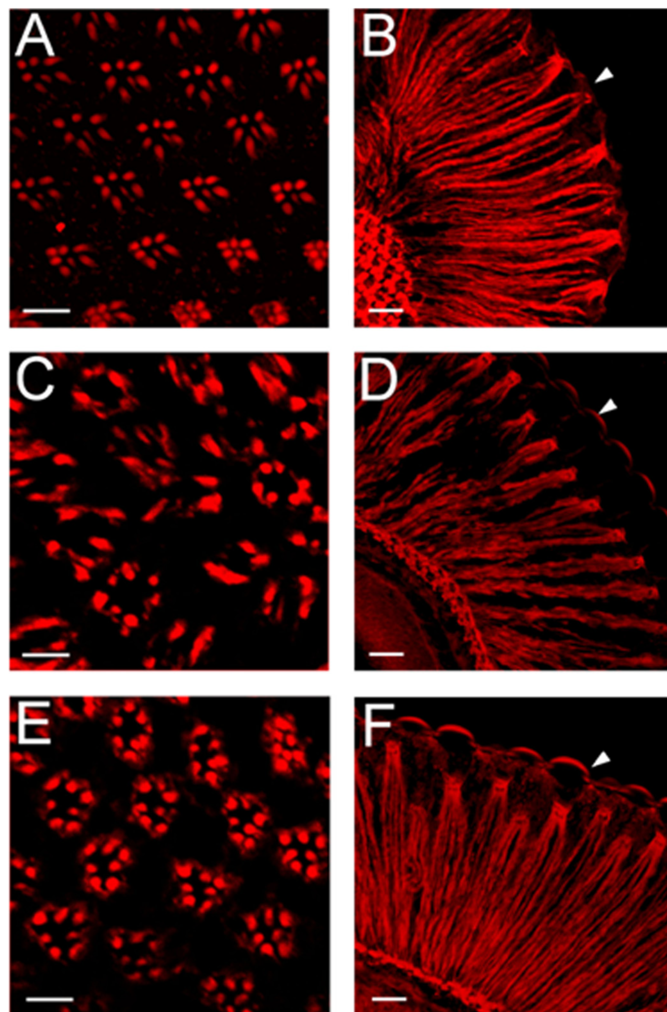

FIGURE 8. **Retinal degeneration is prevented in a chromophore- and opsin-deficient double mutant.** *A–F*, F-actin staining of cryo-sections of the eyes of 3 days post eclosion wild type (*A* and *B*), *ninaE*^{17ol} mutant (*C* and *D*) and *ninaD*^{P245}; *ninaE*^{17ol} double mutant (*E* and *F*) flies. *A*, *C*, and *E*, cross-sections. *B*, *D*, and *F*, longitudinal sections. *Arrowheads* indicate the position of the cornea. *Scale bars* in *A*, *C*, and *E*, 5 μm; in *B*, *D*, and *F*, 10 μm.

pendent manner (Fig. 9, *A–C*). Cells treated with the same amount of all-*trans*-retinol remained viable, suggesting that chromophore toxicity depends on the aldehyde group. To provide *in vivo* data that the chromophore can cause retinal degeneration, we treated adult *ninaE*;*ninaD* double mutants with all-*trans*-retinal. All-*trans*-retinal can bypass defects in chromophore production in adult *ninaD* mutant flies when applied in very high amounts (12, 14). After supplementation with all-*trans*-retinal, flies were kept for 3 days in a light/dark cycle to allow light-dependent chromophore synthesis from this precursor. Photoreceptor ultrastructure was then analyzed by electron microscopy. The all-*trans*-retinal-treated double mutants developed retinal degeneration. Their rhabdomeric membranes involuted into the photoreceptor cell lumen (Fig. 9*E*). In contrast, double mutants treated only with solvent displayed intact rhabdomeres (Fig. 9*D*). These results support the idea that the chromophore is the causative agent for retinal degeneration in opsin-deficient photoreceptors.

DISCUSSION

Successful production of visual pigments is essential for photoreceptor morphogenesis and function. These G-protein-coupled receptors consist of a protein moiety (opsin) and a covalently linked 11-*cis*-retinylidene chromophore. In mammals, RPE65 catalyzes *trans*-to-*cis* isomerization of retinoids, the key step for chromophore production and recycling (3). RPE65 belongs to the same enzyme family as mammalian carotenoid-oxygenases and insect *NinaB*. Recently, we biochemically demonstrated that *NinaB* combines the activities of a retinoid isomerase and a carotenoid-oxygenase in a single protein (25). Here we show that, instead of retinoids and RPE65 as in mammals, it is carotenoids and NinaB that are essential for visual pigment production in *Drosophila*. *ninaB* mutant flies exhibit impaired chromophore production and consequently lack visual pigments. However, they do not show signs of retinal degeneration as described for opsin-deficient photoreceptors. This observation can be explained by our demonstration that retinal degeneration is caused by chromophore itself in opsin-deficient *ninaE* photoreceptors. Retinal degeneration can be prevented by restricting chromophore supply as shown in an opsin and chromophore-deficient double mutant. Thus, our study establishes NinaB as a key component for *Drosophila* vision and provides evidence that NinaB-dependent chromophore production induces retinal degeneration in opsin-deficient photoreceptors.

Chromophore Production Is Eye-dependent—In vertebrates, BCMO1 converts carotenoids to those retinoids required for vision and gene regulation. The resulting retinoids are then transported, taken up by target tissues, and metabolized to reti-

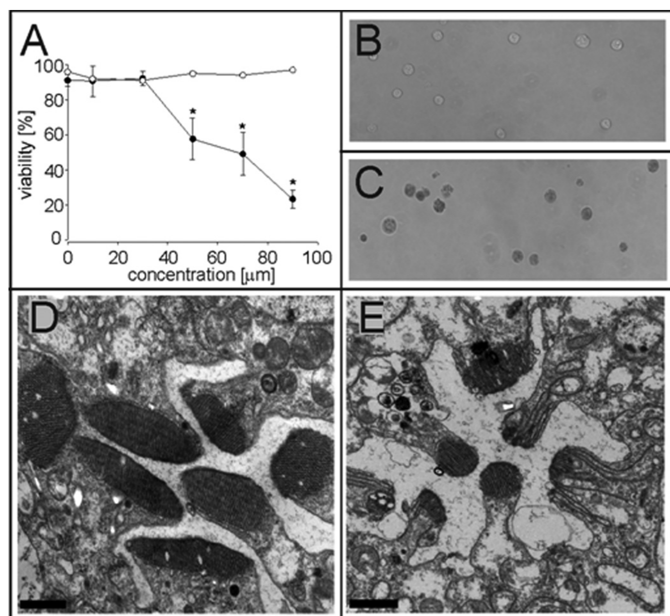

FIGURE 9. **All-*trans*-retinal causes cell damage in S2 cells and opsin-deficient photoreceptors.** *A*, tests for viability of *Drosophila* S2 cells treated with different concentrations of all-*trans*-retinal and all-*trans*-retinol. *B* and *C*, Trypan blue staining of S2 cells treated with all-*trans*-retinal (*B*) and all-*trans*-retinol (*C*). Retinaldehyde concentrations of 50 μM and higher led to a significant (*, $p \leq 0.01$) reduction of cell viability. Treatment with all-*trans*-retinol had no influence on cell viability. *D* and *E*, electron micrographs of cross-sections of the eyes of the *ninaD*^{P245};*ninaE*^{17ol} double mutant. Flies were supplemented with either ethanol (*D*) or 200 μM all-*trans*-retinal dissolved in ethanol (*E*). *Scale bar*, 1 μm.

nyl esters for storage or to retinoic acid for gene regulation. Retinoids in the eyes also are isomerized by RPE65 for chromophore production and regeneration. *Drosophila* require retinoids only for vision (11), and NinaB combines the activity of its mammalian counterparts in a single protein (25). During embryogenesis, NinaB was expressed in the developing BO, and behavioral testing showed that light reception was impaired in *ninaB* mutant larvae. HPLC analysis in *so* flies that lack compound eyes revealed that carotenoids accumulated, but retinoids were absent. RT-PCR analysis additionally indicated that *NinaB* expression was highly reduced in this mutant. This result is consistent with the outcome of a genome-wide microarray analysis that revealed a strong reduction of *ninaB* expression in this mutant (32). Additionally, we found that *ninaB* expression was induced in the thoraces of flies that developed ectopic eyes. These structures, being comprised of fully differentiated light-sensitive photoreceptors, must have produced visual pigments (27). Thus, *ninaB* expression and chromophore production is restricted to the locus of retinoid action in the eyes.

Previous genetic studies ruled out all retinal cells, including photoreceptors and pigment cells, as the site of NinaB action (16, 38). Instead, immunolocalization of a GFP reporter expressed under control of the *ninaB* promoter indicated neuronal and glial cells of the lamina and medulla of the optic lobes as the locus of NinaB action (16). This observation is compatible with the marked reduction in *ninaB* expression in *so* mutant flies, because not only their compound eyes but also the lamina of their optic lobes are greatly reduced (39). Such chromophore production in glial and neuronal cells appears evolutionally conserved. Ganglion cells of the vertebrate retina can also be light-sensitive and express melanopsin (40), the closest vertebrate homolog of insect visual pigments. Additionally, Müller glial cells of the vertebrate retina have been implicated in visual chromophore production for cone photoreceptors (41).

A Carotenoid-dependent Pathway for Chromophore de Novo Synthesis—Carotenoids and retinoids are highly lipophilic molecules, suggesting that specific membrane transporters and binding proteins exist that mediate the transport of these compounds within the body. In *Drosophila*, specific transporters for carotenoids have been identified that mediate absorption of dietary carotenoids (14, 16, 29). The *ninaD* gene encodes a scavenger receptor essential for the accumulation of dietary carotenoids at larval stages (29). During photoreceptor morphogenesis, acquired carotenoids are transported to *ninaB*-expressing cells in a process that depends on the Santa Maria protein (16). However, the fly genome does not encode molecular components for the retinoid transport systems described in vertebrates, *e.g.* the serum retinol-binding protein RBP4 and its receptor STRA6 (42, 43). Only carotenoids such as zeaxanthin and β-carotene consistently support visual pigment production during compound eye development. A previous study showed that β-carotene is converted to zeaxanthin, indicating that this xanthophyll is directly cleaved to 3-hydroxy-retinoids (35). Using a recently established protocol (25), we now show that *Drosophila* NinaB can convert zeaxanthin to 3-hydroxy-retinoids. Thus, hydroxylated carotenoids are the direct precursors for chromophore production. In contrast, preformed retinoids failed to support this process in *Drosophila*, even though all-*trans*-retinal was supplemented in 100-fold higher amounts relative to carotenoids. This inability to transport retinoids was also evidenced by the phenotype resulting from ectopic *ninaB* expression. When we expressed UAS-*ninaB* under a tub-GAL4 driver, ectopic production of 3-hydroxy-retinoids reduced the levels of carotenoids available for chromophore production in the eye with consequent impairment of visual pigment biogenesis. Only when applied in very high amounts to chromophore-deficient adult flies can all-*trans*-retinal be utilized for visual pigment production (4, 14). But this effect relies on nonspecific diffusion of these compounds rather than on a protein-facilitated transport process. When we applied a solution of all-*trans*-retinal directly on the eyes of *ninaB* mutants, visual pigment production was restored in a light-dependent manner. Physiologically, this light-dependent isomerization of retinoids likely plays a role in the conversion of the all-*trans*-stereoisomer produced by carotenoid conversion via NinaB. As recently shown, NinaB converts one half-site of a carotenoid to 11-*cis*-stereoisomer, whereas the other half-site exists in the all-*trans* configuration (25). Accordingly, 11-*cis*-3-hydroxy-retinal and all-*trans*-3-hydroxy-retinal should have existed in equimolar amounts in carotenoid-supplemented dark-reared flies. In illuminated flies, this ratio was altered in favor of the 11-*cis*-3-hydroxy-retinal stereoisomer. The light-dependent pathway for all-*trans* to 11-*cis* isomerization ensures that both retinoid cleavage products can be used for chromophore production.

Although retinoids are not transported well within the *Drosophila* body, the retinoid-binding protein Pinta is evidently required for visual pigment production. Pinta is expressed in the pigment cells and is a homolog of the vertebrate CRALBP that acts downstream of RPE65 (36). Previously, Pinta was suggested to support reduction and oxidation of retinoids for visual chromophore production by binding all-*trans*-retinol (36). However, our biochemical analysis showed that *pinta* mutant flies showed a similar 3-hydroxy-retinoid composition as wild-type flies and that all-*trans*-retinol is absent in flies. Thus, the endogenous 3-hydroxy-retinoid that binds to Pinta remains to be determined. Because *pinta* mutants produced chromophore, but Rh1 levels were highly reduced, Pinta is likely required for the transport of chromophore from its place of NinaB-dependent production to photoreceptor cells.

Coordinated Production of the Chromophore and the Opsin Is Required for Photoreceptor Morphogenesis—In flies, the chromophore is of importance not only for phototransduction but also for targeting of Rh1 to rhabdomere membranes via the secretory pathway (19). Because rhabdomeres of opsin-deficient mutant degenerate (4), we were interested in the consequences of the *ninaB* null mutation for photoreceptor structure. Surprisingly, we found that NinaB-deficient photoreceptors were structurally intact. In *ninaB* flies, some photoreceptors developed two instead of one rhabdomere. This abnormality might be attributed to a requirement of Rh1 in the organization of the cortical actin cytoskeleton during rhabdomere formation in photoreceptor cell development (10), but it is clearly distinguishable from retinal degeneration described for mutants that lack the opsin protein (9). Because we used the *ninaB*^{360d} null mutant, we can exclude the possibility that residual amounts of chromophore prevented retinal degeneration. We further confirmed by immunocytochemistry that opsin was not transported to rhabdomeres in this mutant. The absence of opsin at rhabdomere membranes is in accord with results from the chromophore-deficient *ninaG* mutant (15). We conclude that functional Rh1 is not required for structural integrity of rhabdomeres.

Because lack of opsin is not the proximal cause of retinal degeneration, we speculated that chromophore is the causative agent. It has been previously shown that retinal degeneration in the *ninaE* mutant can be prevented by transient expression of a wild-type *ninaE* transgene at the late pupal stage (44) when *ninaB* expression and chromophore production is initiated during eye development (29). A need to coordinate opsin and chromophore production is also evidenced by our finding that photoreceptor degeneration in a *ninaE* null mutant can be rescued by genetically induced chromophore deficiency. Conversely, supplementation with high amounts of all-*trans*-retinal of the adult *ninaE/ninaD* double mutant did result in retinal degeneration. Dose-dependent toxicity of all-*trans*-retinal was also found in *Drosophila* S2 cells. No such effect was found when cells were incubated with similar amounts of all-*trans*-retinol. These results implicate the aldehyde functional group of the chromophore in this toxicity. This agrees with the known chemistry of retinaldehyde. It can form Schiff base adducts with phosphatidylethanolamine, which in turn can react with a second retinal molecule to produce *N*-retinylidene-*N*-retinyl ethanolamine, which has been implicated in the pathogenesis of certain human retinal diseases (45). In addition to forming toxic adducts, depletion of components such as phosphatidylethanolamine from rhabdomeres may interfere with the structural integrity of these membranes. Finally, reactivity of an aldehyde might also lead to interactions with other cell components, including amino groups of metabolic intermediates, DNA, and proteins. Such toxicity has been recently suggested to contribute to retinal damage in mammals under conditions wherein the all-*trans*-retinal photoproduct accumulates (46). Accordingly, abolition of chromophore production in mammals prevents light-induced retinal degeneration (47). HPLC analysis revealed that *ninaE* mutants produced chromophore despite opsin deficiency, although overall retinoid content was reduced as compared with wild-type controls (supplemental Fig. S1). Thus, retinaldehyde toxicity is likely an immediate cause of the retinal degeneration in *Drosophila* as well.

In summary, our analysis establishes NinaB as a key component for visual pigment production and vision in *Drosophila*. For chromophore production, carotenoids rather than retinoids must be transported to *ninaB*-expressing neuronal and glial cells in the lamina of the optic lobes. NinaB then converts carotenoids directly to the chromophore in a combined isomerization-oxygenase reaction. Downstream in the pathway, the retinoid-binding protein Pinta is then required to supply photoreceptors with the chromophore. Toxicity resulting from lack of opsin production is in turn not due to a structural requirement of Rh1 for rhabdomere biogenesis, but rather to the resulting free chromophore. Both *ninaE* and *ninaB* are governed by *so* and *ey*, major control factors for eye development. This spatiotemporal coordination of chromophore and opsin production optimizes visual pigment biogenesis and protects the structural integrity of photoreceptors from excess chromophore. Thus, our findings open the possibility of using *Drosophila* mutants to screen for genetic suppressors and drugs that reduce the retinal degeneration and blindness resulting from retinaldehyde toxicity.

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