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Septate junctions are required for ommatidial integrity and blood–eye barrier function in *Drosophila*

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ABSTRACT

The anatomical organization of the *Drosophila* ommatidia is achieved by specification and contextual placement of photoreceptors, cone and pigment cells. The photoreceptors must be sealed from high ionic concentrations of the hemolymph by a barrier to allow phototransduction. In vertebrates, a blood–retinal barrier (BRB) is established by tight junctions (TJs) present in the retinal pigment epithelium and endothelial membrane of the retinal vessels. In *Drosophila* ommatidia, the junctional organization and barrier formation is poorly understood. Here we report that septate junctions (SJs), the vertebrate analogs of TJs, are present in the adult ommatidia and are formed between and among the cone and pigment cells. We show that the localization of Neurexin IV (Nrx IV), a SJ-specific protein, coincides with the location of SJs in the cone and pigment cells. Somatic mosaic analysis of *nrx IV* null mutants shows that loss of Nrx IV leads to defects in ommatidial morphology and integrity. *nrx IV* hypomorphic allelic combinations generated viable adults with defective SJs and displayed a compromised blood–eye barrier (BEB) function. These findings establish that SJs are essential for ommatidial integrity and in creating a BEB around the ion and light sensitive photoreceptors. Our studies may provide clues towards understanding the vertebrate BEB formation and function.

Introduction

The *Drosophila* compound eye is composed of about 800 units, the ommatidia, which are arranged in a hexagonal array to produce a stereotyped pattern. Each ommatidium consists of eight photoreceptors (PRs), four cone cells (CCs) and three types of pigment cells (PCs) (Tomlinson and Ready, 1987; Cagan and Ready, 1989; Wolff and Ready, 1991a; reviewed in Wolff and Ready, 1993). The eye develops from a single layer of proliferative epithelial cells, the eye imaginal disc, which is initially unpatterned during the first and second larval instar stages. Differentiation begins from the mid-third instar and is controlled by genes such as *eyeless*, *dachshund*, *sine oculis* and *eyes absent* (Chen et al., 1997; Halder et al., 1998; Mardon et al., 1994; Wolff, 2003). An indentation of the epithelium, called the morphogenetic furrow, forms in the third-instar eye imaginal disc. Anterior to the furrow, the cells divide asynchronously while posterior to the furrow, the cells begin to arrange into evenly spaced ommatidial precursor clusters and first differentiate into PR neurons (Tomlinson and Ready, 1987; Cagan and Ready, 1989; Wolff and Ready, 1991a). The non-neuronal CCs are added to each ommatidial cluster after the specification of the PRs in the late third instar eye disc (Tomlinson, 1988). The PCs and the sensory units (bristles) are recruited during the pupal stage, thereby completing the ommatidial assembly (Cagan and Ready, 1989; Wolff and Ready, 1993). Recent studies on the phenotypic characterization of *sparkling*, *bar*, and *cut* mutants have revealed important aspects of CC and/or PC functions during eye development (Blochlinger et al., 1993; Fu and Noll, 1997; Hayashi et al., 1998; Higashijima et al., 1992). However, CCs and PCs have received much less attention compared to the more extensively studied PRs, thus leading to gaps in our understanding of their range of functions.

The eye serves as a visual transduction machinery to perform photoreception and transduction functions in both vertebrates and invertebrates. In vertebrates, the retina contains two types of specialized PRs: rods and cones. The rods are more numerous than cones but both contain stacks of membranous discs which primarily perform the photoreception and phototransduction (Smith, 2002). The vertebrate retinal pigment epithelium forms an outer blood–retinal barrier (BRB) by separating the neural retina from the fenestrated capillaries in the choroids. This barrier depends upon TJs within the apical junctional complexes that bind neighboring cells (Williams and Rizzolo, 1997). Recent studies on the BRB in vertebrate models and human

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have highlighted its importance in many human ocular disorders, such as macular edema, diabetic retinopathy and retinitis pigmentosa (Vinores et al., 1999). One of the major causes of a BRB dysfunction in vertebrates is an increased permeability and/or structural alterations of the occluding TJs (Peng et al., 2003).

The PRs that form the core of the *Drosophila* ommatidium are housed within specially designed subcellular compartments that serve to maximize the amount of light absorbing membranes and molecules (Hardie and Raghu, 2001; Kumar and Ready, 1995). The PRs have densely packed microvilli forming the cylindrical rhabdomeres that form the seat of the phototransduction cassette as the rhabdomere membrane contains large quantities of light sensitive opsin and channel proteins (Hardie and Raghu, 2001; Ranganathan et al., 1995). For phototransduction to occur, these channel proteins and the PR units need to be protected from the circulating hemolymph, thereby raising a strong possibility of the existence of a protective blood–eye barrier (BEB) in the *Drosophila* retina. A detailed description of various forms of cell–cell junctions in *Drosophila* embryos (Tepass and Hartenstein, 1994), *Musca domestica* (reviewed in Carlson et al., 2000) and other insects (Lane, 2001) provided insights into the various forms of junctions in insects and their putative functions. Although TJs in *Drosophila* embryos and larvae have never been observed (Tepass and Hartenstein, 1994; Carlson et al., 2000; Tepass et al., 2001), fly homologs of vertebrate TJ proteins, Claudins, are present in *Drosophila* and localize to septate junctions (SJs) in embryonic epithelial cells (Behr et al., 2003; Wu et al., 2004) and glial cells (Banerjee and Bhat, unpublished data). SJs are a specialized form of cell–cell junctions that display a characteristic electron-dense ladder-like structure, hence the name septate junctions, and have been shown to perform a barrier function in epithelia and glial cells (Auld et al., 1995; Banerjee and Bhat, 2007; Banerjee et al., 2006; Baumgartner et al., 1996; Faivre-Sarrailh et al., 2004). However, the presence of SJs in the adult *Drosophila* ommatidia or the cell types that might establish these junctions are still unknown.

Here, we report that SJs are present in the adult *Drosophila* ommatidia between and among the CCs and PCs. Immunofluorescence localization of a SJ-specific marker, Neurexin IV (Nrx IV) correlates with the ultrastructural presence of SJs in the developing and adult ommatidia. Using somatic clonal analysis, we demonstrate that Nrx IV function is essential for the maintenance of the ommatidial integrity in the developing and adult *Drosophila* eye. In addition, we show that viable hypomorphic *nrx IV* mutant combinations have altered distribution of Nrx IV in the adult CCs and PCs and a compromised BEB function.

Materials and methods

Drosophila stocks

Canton S strain was used as wild type. All *nrx IV* alleles used in this study have been previously reported in Baumgartner et al. (1996). All other fly reagents used in this study were obtained from the Bloomington Stock Center, Indiana.

Histology, scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Histology, fixation, sectioning, and SEM of adult eyes were carried out according to published procedures with minor modifications (Cagan and Ready, 1989; Longley and Ready, 1995). Briefly adult eyes or pupal or larval discs were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 3 h at room temperature followed by three 5 min washes in 0.1 M sodium cacodylate buffer, pH 7.2. The tissues was postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 h followed by three 5 min washes in deionized water. The tissues were then dehydrated in 30, 50, 75, and 95% ethanol for 10 min each followed by two 10 min steps in absolute ethanol and propylene oxide. The tissue was then infiltrated with 1:1 propylene oxide and Spurr's resin in propylene oxide for 3 h followed by overnight infiltration in 100% Spurr's resin. The tissue was embedded in flat molds in 100% Spurr's resin for 24 h at 70 °C. For initial light microscopic analysis and depth of the tissues, 1 μm thick sections were cut with a glass knife, mounted on a glass slide and stained with 1% toluidine blue in 1% sodium borate for 30 s at 60 °C. Once the desired area of the tissues was identified 60–70 nm ultrathin sections were cut with a diamond knife and mounted on a 200 or 300 mesh copper grids, and double stained with 4% aqueous uranyl acetate for 15 min, followed by Reynolds' lead citrate for 7 min. The grids were washed in deionized water and allowed to dry at room temperature and analyzed on LEO EM 910 transmission electron microscope equipped with a high-resolution digital camera. All images were then processed with Adobe Photoshop Software.

Immunohistochemistry

Immunostaining of imaginal discs, pupal and adult eyes was done as previously described (Izaddoost et al., 2002). Primary antibodies used for immunofluorescence staining were: rabbit anti-Nrx IV (Baumgartner et al., 1996); guinea pig anti-Dlg (P. Bryant, UC, Irvine), rat anti-Crumbs (Bhat et al., 1999); mouse anti-Cadherin (Oda et al., 1994); mouse anti-Elav (DSHB, Iowa) and mouse anti-chaoptin (DSHB, Iowa). All secondary antibodies used in this study were from Jackson Immunochemicals. Images were captured on a BioRad Radiance 2000 confocal microscope and processed with Adobe PhotoShop software.

Somatic mosaic analysis in nrx IV mutants

Clones of cells lacking *nrx IV* were generated by *FLP*-mediated mitotic recombination with two *nrx IV* null alleles by subjecting *yw, hsp70-flp/+; nrx IV^{4304}, FRT80B/w^{+}FRT80B* or *yw, hsp70-flp/+; nrx IV^{4025},FRT80B/w^{+}FRT80B* larvae 24–48 h after egg lay to a single heat shock at 38.5 °C in a water bath for 1 h and 30 min (Xu and Rubin, 1993). For adult eye clonal analysis, clones were visually identified and the eyes were processed for SEM or TEM as described above. For TEM analysis at least 3 large and 5 small independent clones were processed from adults of two *nrx IV* null alleles (*nrx IV^{4304}* and *nrx IV^{4025}*) (Baumgartner et al., 1996). For light microscopic phenotypic evaluation, at least 5 independent eyes with large mutant clones (covering a space of more than 10 ommatidia) from *nrx IV* null alleles (*nrx IV^{4304}* and *nrx IV^{4025}*) were analyzed. Depending upon the area and the extent of the *nrx IV* mutant clone size, we performed either longitudinal or cross sectional analysis. Cross section clones were analyzed within a depth anywhere between 15 and 30 μm at 70 nm thickness and longitudinal clones were analyzed within a depth of 10 μm at 70 nm thickness.

Sequence analysis of nrx IV alleles

For sequencing of the genomic DNA by PCR, manufacturer's instructions were followed and wherever necessary standard molecular biology protocols were used. Overlapping M13forward- and M13reverse-extended primers which spanned the entire genomic region of *Drosophila NRX IV* were as follows:

F102-TGTAAA ACGACGGCCAGTGGAGTGTAAAGTGCACTCC,
F460-TGTAAAACGACGGCC AGTCGTCCTCACGGCCACATCATC,
R708-CAGGAAACAGCTATGACCTAGAGTT GCCATCGGAGTTG,
F837-TGTAAAACGACGGCCAGTCTCTACGGTTGCGAT TAC,
R1337-CAGGAAACAGCTATGACCGAAGAGATATCCCTCTCC,
F1224-TGTA AAACGACGGCCAGTGGGCGCTGTATCTAGGTGGTG,
R2109-CAGGAAACAGCT ATGACCGGTCACTGGGAAAGGTTCTAG,
F2015-TGTAAAACGACGGCCAGTGG AGCTGTTTGCCATACA,
R2495-CAGGAAACAGCTATGACCTCCAGACTGTTCG AATCG,
F2852-TGTAAAACGACGGCCAGTTCCGTCCCTTCTCTTGGTGGA,
R3221 CAGGAAACAGCTATGACCACGGCAATCGCAGCTGTAGCC,
F3108-TGTAAAACG ACGGCCAGTGCACTGCTGCTGAACGGAAAG,
R3815-CAGGAAACAGCTATGAC CTGGCTCCACTCCGCAGAAATC,
F3656-TGTAAAACGACGGCCAGTCTCAGC GGATGCTCAATTC, and
R4171-CAGGAAACAGCTATGACCTCGCTGTTGCGTG TCTAAG.

Adult fly dye injection assay, confocal images of dissected fly brains and quantitation of dye leakage

Adult flies were hemolymph injected with 100 nl of 50 mg/ml 3 kDa FITC-Dextran dye (Molecular Probes). Retinal fluorescent images were acquired after overnight recovery as described in Bainton et al. (2005). Briefly, after injection flies were allowed to recover overnight and anesthetized with CO_2 and placed in cold phosphate buffered saline (PBS, pH 7.2). The heads were removed manually with forceps and proboscis removed by pulling outwards and quickly placed in 3.7% paraformaldehyde and incubated for 15 min at room temperature. Brains were then dissected from the cuticle leaving the retina intact in cold PBS and transferred to 0.5 ml PBS and then to mounting media (DakoCytomation Fluorescent Mounting medium) for 10 min. Brains were mounted retinal side down on glass slides, covered with media and a coverslip and sealed with clear nail polish. At this stage brains were either immediately processed for confocal analysis or stored at −20 °C for up to a week before confocal analysis. Confocal images were acquired on a Zeiss LSM510 confocal microscope and images were taken at 16–21 μm depth from the brain surface with a 40× H_2O objective. Laser and detector gains were established based on wild type animal's internal brain auto-fluorescence. A square of 1656 pixels was drawn on different parts of the wild type brain parenchyma image and gains were set to maintain a fluorescence intensity of 3–7 units per 1656 pixels on the wild type animals. Images of *nrx IV* hypomorph brains were taken at the same confocal settings, depth of section and anatomic location. Pixel intensities for squares of 1656 pixels were take in different parts of the mutant brains to establish a range of fluorescence intensity for each hypomorphic combination. Fluorescent dye