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Loss of Na^+/K^+-ATPase in *Drosophila* photoreceptors leads to blindness and age-dependent neurodegeneration

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ABSTRACT

The activity of Na^+/K^+-ATPase establishes transmembrane ion gradients and is essential to cell function and survival. Either dysregulation or deficiency of neuronal Na^+/K^+-ATPase has been implicated in the pathogenesis of many neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and rapid-onset dystonia Parkinsonism. However, genetic evidence that directly links neuronal Na^+/K^+-ATPase deficiency to in vivo neurodegeneration has been lacking. In this study, we use *Drosophila* photoreceptors to investigate the cell-autonomous effects of neuronal Na^+/K^+ ATPase. Loss of ATPα, an α subunit of Na^+/K^+-ATPase, in photoreceptors through UAS/Gal4-mediated RNAi eliminated the light-triggered depolarization of the photoreceptors, rendering the fly virtually blind in behavioral assays. Intracellular recordings indicated that ATPα knockdown photoreceptors were already depolarized in the dark, which was due to a loss of intracellular K^+. Importantly, ATPα knockdown resulted in the degeneration of photoreceptors in older flies. This degeneration was independent of light and showed characteristics of apoptotic/hybrid cell death as observed via electron microscopy analysis. Loss of Nrv3, a Na^+/K^+-ATPase β subunit, partially reproduced the signaling and degenerative defects observed in ATPα knockdown flies. Thus, the loss of Na^+/K^+-ATPase not only eradicates visual function but also causes age-dependent degeneration in photoreceptors, confirming the link between neuronal Na^+/K^+ ATPase deficiency and in vivo neurodegeneration. This work also establishes *Drosophila* photoreceptors as a genetic model for studying the cell-autonomous mechanisms underlying neuronal Na^+/K^+ ATPase deficiency-mediated neurodegeneration.

Introduction

The Na^+/K^+-ATPase transports Na^+ and K^+ against their concentration gradients across the cell membrane to maintain a low Na^+ and high K^+ concentration within the cells (Blanco and Mercer, 1998; Mobasheri et al., 2000). These ion gradients determine the resting membrane potential and form the basis of the excitability of neurons. The Na^+ gradient also provides the driving force for various secondary active transporters that import glucose, amino acids, and other nutrients into the cell. Additionally, the ion concentrations maintained by Na^+/K^+-ATPase are important for regulating cellular volume and preventing cells such as neurons from swelling and lysing (Geering, 1997; Pavlov and Sokolov, 2000).

Considering the importance of Na^+/K^+-ATPase in basic cellular functions, it is not surprising that either dysregulation or deficiency of neuronal Na^+/K^+-ATPase was observed in many neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD) and rapid-onset dystonia Parkinsonism (RDP) (Cannon, 2004; Chauhan et al., 1997; de Carvalho Aguiar et al., 2004; DeAndrade et al., 2011; Kumar and Kurup, 2002). Thus, disrupting normal Na^+/K^+-ATPase activity in neurons has been proposed to contribute to the pathogenesis of neurodegeneration. Nevertheless, the link between the disrupting neuronal Na^+/K^+-ATPase activity and neuronal dysfunction/degeneration has yet to be clarified.

Na^+/K^+-ATPase is composed of at least two subunits: a large catalytic α subunit and a regulatory, single-transmembrane-domain β subunit (Gloor et al., 1990; Horisberger, 2004; Kaplan, 2002; Paul et al., 2007; Shoshani et al., 2005; Vagin et al., 2005). Mammals have three α-subunit and two β-subunit genes and may express six structurally distinct Na^+/K^+-ATPase isoforms (Watts et al., 1991). In the brain, although the α3 and β2 subunits are expressed predominantly in neurons, the α2 and β1 subunits are found primarily in glia, and the α1 subunit is ubiquitously expressed (McGrail et al., 1991; Watts et al., 1991). The Na^+/K^+-ATPase in glia is required to maintain a low K^+ level in the neuronal environment (Wang et al., 2012) and thus has a large impact on neuronal function and survival. Na^+/K^+-ATPase inhibitors like ouabain act on all Na^+/K^+-ATPase

Abbreviations: AD, Alzheimer's disease; PD, Parkinson's disease; RDP, Rapid-onset dystonia Parkinsonism; ERG, Electroretinograms; GFS, Giant fiber system.

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isoforms and cannot differentiate between the cell-autonomous effects of the Na^+/K^+-ATPase in neurons from those derived from the neighboring glia. Thus, genetic approaches are needed to modulate the Na^+/K^+-ATPase level in neurons to investigate the function of neuronal Na^+/K^+-ATPase. Genetic studies on the impact of neuronal Na^+/K^+-ATPase deficiency in the past decade, which were mostly based on characterization of heterozygous mutant mice of the α3 subunit, have identified defects in the function of central brain neurons (Clapcote et al., 2009; Moseley et al., 2007; Shiina et al., 2010) but have not provided direct evidence of neurodegeneration.

The *Drosophila* visual system expresses only one type of α subunit, ATPα, and three β subunits, Nrv1–3 (Ashmore et al., 2009; Baumann et al., 2010; Okamura et al., 2003; Palladino et al., 2003; Takeyasu et al., 2001). In this study, we used *Drosophila* photoreceptors as a genetic model to study the cell-autonomous functions of neuronal Na^+/K^+-ATPase. Although ATPα mutants in *Drosophila* exhibit extensive neurodegeneration (Palladino et al., 2003), these mutants were not used because the degeneration is due to the loss of Na^+/K^+-ATPase not only in neurons but also in neighboring non-neuronal cells. Instead, using a UAS/Gal4-mediated RNAi approach (Brand and Perrimon, 1993; Dietzl et al., 2007; Roy et al., 2013), we knocked down ATPα and Nrv1–3 specifically in photoreceptors and assessed the impact of this knockdown on visual signaling and photoreceptor integrity in the fly.

Materials and methods

Drosophila stocks and crosses

All flies were raised on corn-meal medium without propionic acid and were maintained at 25 °C and 60% humidity under a 12:12 h light–dark cycle unless otherwise stated. The following fly stocks were used: repo-Gal4, elav-Gal4, lGMR-Gal4 (longGMR, pan-photoreceptor-Gal4, BL8605), GMR-Gal4 (ninaE.GMR-Gal4, BL1104), UAS-ATPα-RNAi (short-hairpin, BL33646), UAS-nrv3-RNAi (BL29431) and tublin-Gal80^{ts}, all of which were obtained from the *Drosophila* Stock Center in Bloomington. The fly stocks UAS-ATPα-RNAi (v100619), UAS-nrv1-RNAi (v103702) and UAS-nrv2-RNAi (v2660) were also used and supplied by the Vienna *Drosophila* RNAi Center. UAS-RNAi flies were crossed over specific GAL4 and Gal80^{ts} to either induce or inhibit the expression of RNAi, respectively.

The RNAi constructs of the Na^+/K^+-ATPase subunit genes were expressed in photoreceptors using the Gal4/UAS system (Brand and Perrimon, 1993; Dietzl et al., 2007; Roy et al., 2013). The drivers lGMR-Gal4 (Chen et al., 2014; Timofeev et al., 2012; Wernet et al., 2003) and GMR-Gal4 (Velentzas et al., 2013) have a photoreceptor-specific expression pattern, and *repo*- (Awasaki and Ito, 2004) and *elav*-Gal4 (Zhan et al., 2004) have glial and neuronal expression patterns, respectively. The Gal80ts/TARGET system was used for temporal control of UAS-RNAi expression (McGuire et al., 2004).

Electrophysiological recordings

Electroretinograms (ERG) were recorded as previously described (Li and Montell, 2000). Flies were immobilized with thin strips of tape. Glass recording microelectrodes filled with Ringer's solution were placed on the eye surface of the fly. A second extracellular recording electrode was maintained on the thorax (as a reference). Five-second orange light pulses (4000 lx) were used to stimulate the eye after adapting the fly to the dark for 1 min. The signal was amplified and recorded using a Warner IE210 intracellular electrometer.

In vivo photoreceptor intracellular recordings were performed as previously described (Johnson and Pak, 1986). Briefly, a small portion of the cornea was removed with a sharp needle, and the opening was covered with Vaseline petroleum jelly. The intracellular recording electrodes were inserted into the retina through this opening. The recording electrodes had a resistance of 100–150 MΩ when filled with 4% neurobiotin (Vector Labs) in 2 M potassium acetate (KAc). The reference electrode was filled with Ringer's solution, and its tip was placed in the photoreceptor layer. The fly was dark-adapted for 10 min before measurement. Voltage responses were amplified using a Warner IE210 intracellular electrometer in current clamp mode. When the electrode was inserted into a cell, we measured the resting membrane potential in the dark based on a sudden increase of capacitance and tested the cell's response to 5 s orange light pulses (4000 lx). After the recording, the cell was injected with neurobiotin by passing 1 nA depolarizing rectangular pulses at 1 Hz for 5 min (Kita and Armstrong, 1991). The retina was subsequently dissected, fixed in 4% paraformaldehyde and stained with streptavidin-Alexa Fluor 488 conjugate (Invitrogen) and rhodamine phalloidin to confirm the photoreceptor identity of the recorded cell (Schnell et al., 2010).

Immunofluorescence staining

For cryosectioning, 1-day-old fly heads were removed, incubated in 0.1 M phosphate buffer with increasing concentrations of sucrose, infiltrated and embedded in TFM tissue freezing medium (Ted Pella Inc.). Approximately 20 μm sections were cut at −20 °C and subjected to immunofluorescence staining. After a 30 min incubation with blocking buffer (5% fetal bovine serum in PBS containing 0.3% Triton X-100), the brain sections were incubated overnight at 4 °C with primary antibody diluted in blocking buffer. After three washes in PBS containing 0.3% Triton X-100, the brain sections were incubated with FITC-conjugated secondary antibodies for 3 h at room temperature, washed and mounted in Vectashield medium (Vector Laboratories). The images were captured using confocal microscopy with an LSM 510 instrument (Zeiss). The monoclonal antibody α5-IgG (1:100), which is specific for the α-subunit of the Na^+/K^+-ATPase, was obtained from the Developmental Studies Hybridoma Bank (Baumann et al., 2010; Lebovitz et al., 1989; Takeyasu et al., 1988). We used the auto-fluorescent properties of visual pigments to mark pigment cells in the retina of red eye flies (Pichaud and Desplan, 2001).

Electron microscopy (EM)

Fly heads were removed, bisected and fixed in a solution of 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4), and processed for EM as previously described (Meinertzhagen and O'Neil, 1991). After three washes, fly heads were post-fixed with 2% osmium tetroxide for 5 h, dehydrated in ethanol, infiltrated with propylene oxide and embedded in polybed812 resin (08792-1; Polysciences). After sectioning and staining with uranyl acetate and lead citrate, the ultrastructures in the retina were examined at 80 kV using a Philips Tecnai 12 electron microscope.

Optical neutralization analysis and retinal degeneration assay

This analysis was performed as previously described (Franceschini and Kirschfeld, 1971). Briefly, fly heads were separated from the body and immersed in a layer of lens oil to optically neutralize the cornea. On the microscope stage, a spotlight was shone into the head from the neck side to antidromically illuminate the compound eye. After the images were acquired with a CoolSNAP ES2 CCD Camera, the number of rhabdomeres that appeared as bright dots resulting from a high transmission of light was counted for each upright ommatidium (Sengupta et al., 2013). Degeneration of photoreceptors was assessed directly with this method (Lessing and Bonini, 2009). Briefly, a score ranging from 0 to 3 was given to each rhabdomere depending on the state of its degeneration: 3 was indicative of no degeneration; 0 corresponded to total loss of the rhabdomeres and a score of 1 or 2 suggested partial degeneration. The total score of an intact ommatidium is 21. The rhabdomere integrity index for a fly was the average score from 6 axially