Original Research

Time course of retinal degeneration associated with the absence of 1, 4, 5-inositol trisphosphate receptor in *Drosophila melanogaster*

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Abstract

The absence of the inositol trisphosphate receptor is associated with a gradual retinal degeneration in *Drosophila melanogaster*. To characterize the time-course profile of this process, mosaic flies expressing a null allele of the *itp* gene in the eye were studied by electroretinograms and electronic microscopy. Membrane contour alterations, disrupted mitochondria, altered morphology and even loss of photoreceptors were increased progressively starting 5 d after hatching, were more evident during days 10–15 and promoted highly disorganized structures thereafter. The synaptic transmission and membrane potential of retinal cells were also significantly distorted, showing reduced ON and OFF transients as well as membrane potential from day 10 of hatching, and the functional defects became progressively more severe. Unexpectedly, these alterations were detected not only in the non-pigmented mutant ommatidia, but also in the pigmented ommatidia, including heterozygous and twin clones expressing 1, 4, 5-inositol trisphosphate receptor (IP_3R). To explore the mechanism underlying this degenerative process, the progression of pro-oxidant and apoptotic reactions was characterized by immunohistochemical techniques. Mutant ommatidia showed intermittent episodes of increased pro-oxidant reactions (detected as adducts of 4-hydroxy-nonenal) throughout the fly's life. Similarly, several episodes of active caspase 3, an apoptotic effector, were evident with the same time pattern. Episodes of enhanced lipid peroxidation and apoptosis were also observed in the pigmented ommatidia of the mosaic eyes. The results indicate that photoreceptors lacking IP_3R suffer episodes of increased lipid peroxidation, which eventually perturb the retinal subcellular organization and disrupt the phototransduction process and cell viability. Pigmented ommatidia also showed a similar pattern of damage, indicating that the degenerative process is non-autonomous and is so intense that it propagated to the non-mutant retinal cells in the mosaic eyes. In conclusion, ommatidia with a null mutation of IP_3R degenerate by a process associated with intermittent lipid peroxidation and apoptotic activities.

Keywords: null mutation, apoptosis, oxidative stress, 4-hydroxy-nonenal, electroretinogram, electron microscopy

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Introduction

The 1, 4, 5-inositol trisphosphate receptor (IP_3R) is a high molecular weight, tetrameric protein that participates in calcium dynamics in non-excitable and excitable cells. The IP_3R functions as an intracellular calcium-release channel in response to signal transduction processes. This receptor is an allosteric protein whose activity is modulated by a number of metabolic factors such as calcium levels, adenine nucleotides, redox state, phosphorylation–dephosphorylation and the presence of regulatory proteins, among others.[1,2] In particular, the IP_3R is involved in the control of gene expression, neuronal signaling, exocytosis, intracellular pH and apoptosis. Indeed, experiments in tissues with enhanced or abolished expression of IP_3R have indicated a direct role of this calcium-release channel in cellular duplication and survival.[3] It has been demonstrated that the carboxy-terminal region of the IP_3R binds the proapoptotic mitochondrial

factor cytochrome C[3] as well as the mutant protein huntingtin, which is directly responsible for the neurodegenerative progression linked to Huntington's disease.[4] The association of both of these proteins with IP_3R enhances its response and promotes abnormal calcium signaling prior to the onset of apoptotic activity.[3,4]

The genome of *Drosophila melanogaster* shows only one gene, DroIP_3R, that codes for IP_3R, and it is localized in the right arm of the third chromosome at the position 83A5–83A9. DroIP_3R is expressed ubiquitously and is implicated at several stages of fly development, but the precise role played in specific cellular systems, tissues and organs is still undetermined;[5] DroIP_3R shows 50% homology with IP_3Rs in vertebrates.[6] We reported coincidence between ligand binding parameters such as [^{3}H]-IP_3 affinity and sensitivity to antagonists (heparin, aceto-methoxy 2-aminoethoxydiphenyl borate and xestospongin C) between DroIP_3R and IP_3Rs from vertebrate organisms.[7] In addition, a fluorescent probe specific for DroIP_3R showed positive signal in the cytoplasm of almost every tissue of embryos and adult flies.[7] In 1997, Venkatesh and Hasan[5] reported a DroIP_3R mutant allele constructed by the insertion of a P element, and the homozygotic animals with this mutation (Itp-r83A^{05616}) died at the second larva instar stage.

The *Drosophila* adult eye has ~800 visual units known as ommatidia. Each ommatidium is formed by 20 different cell types, eight photoreceptor and 12 accessory cells. Each photoreceptor shows a microvillar structure called rhabdomere, which is the functional part of the system during the phototransduction process. This event is highly dependent on extracellular Ca^{2+} and is mediated by two principal types of plasma membrane calcium channels: transient receptor potential and transient receptor potential-like, respectively.[8]

Since phototransduction is impaired in flies expressing mutant alleles of phospholipase C in the eye (*norpA*), it was hypothesized that the DroIP_3R might be playing a role in the molecular events underlying the light-driven activation of *Drosophila* photoreceptors.[8] However, this suggestion was excluded based on studies of null mutants for DroIP_3R in retina, in which the phototransduction process was completely normal.[9,10] Strikingly, after a few days, the mutant flies showed a progressive retinal degeneration that severely affected the photoreceptor structure even when the flies were kept in darkness.[9]

Given the close relation between disruption of intracellular calcium dynamics and generation of reactive oxygen species (ROS),[11] this project was based on the hypothesis that the failure to express DroIP_3R in the retina would cause an onset of pro-oxidant reactions. Eventually, the concomitant oxidative stress would interfere with the organization and alter the function of the photoreceptors, leading to cell death.

Indeed, using flies with mosaic eyes, we demonstrated that prior to the structural and functional modifications elicited by the lack of IP_3R, the affected photoreceptors showed episodes of increasing lipid peroxidative activity as well as apoptosis. The extent of the damage was non-autonomous since it was not confined to the mutant ommatidia but extended to the pigmented ommatidia, in accordance with previous reports of some mutations that are so aggressive that their deleterious effects spread to adjacent cells.[12]

Materials and methods

Materials

For immunohistochemical experiments, the antibody anti-caspase 3 was obtained from Cell Signaling Company (Beverly, MA, USA) and anti-4-hydroxy-nonenal (4-HNE) from Alpha Diagnostic International (San Antonio, TX, USA). The resin for electron microscopy was from Electron Microscopy Sciences (Hatfield, PA, USA). Secondary antibodies were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). All the other chemical substances were purchased from Sigma-Aldrich Company (St Louis, MO, USA).

Mutant flies

Mutant flies lacking IP_3R were kindly donated by Dr Padinjat Raghu (Cambridge University, Cambridge, UK) (allele l^3 itpr$^{90B.0}$). A complete molecular characterization of this mutation was previously reported by Venkatesh and Hasan.[5]

Experimental and control groups

Mosaics were generated by the expression of the gene *eyeflp* (yweyflp; FRT82armlacZ/TM6, Ubx) in the mutant flies donated by Dr Raghu.

Fly stocks were maintained on a 12 h light/12 h dark cycle (lights on at 07:00) at constant temperature (≈28°C). We express the mosaic using the FRT90IP_3R^{B0} stock (obtained from Dr Raghu) mating with a yweyflp; FRT82armlacZ/TM6, Ubx stock.[13,14] Flies are mosaics of pigmented (heterozygous or homozygous for the w+ marker) and unpigmented (lacking the marker) tissue.

Non-mosaic, heterozygous flies were used as additional controls. They were born in the same cohort from which mosaics were obtained, and they were selected by their lack of eye pigmentation.

Electroretinograms

Flies were attached to a coverslip with dental wax; the ground electrode was introduced into the thorax, whereas the recording electrode was placed in contact with the eye surface. The electroretinogram was initiated by a light beam of 100 ms, and the recorded activity was collected by an amplifier at 500 ms/20 mV.[15]

Recording was carried out in non-mosaic and mosaic flies. In the mosaic flies, five recordings were made in the pigmented ommatidia section of the eye and another five in the mutant zone (without pigmentation) of each fly. The experiment was performed from 10:00 to 12:00 h in 8–10 flies at each time point studied.

Electron microscopy

Fly heads were dissected and fixed in 0.1 mmol/L cacodylate buffer, pH 7.4, containing 2% OsO_4 and 2.5% glutaraldehyde for 2 h. Dehydration was achieved with ethanol in increasing concentrations: 30%, 50%, 70%, 90% and 100%. After dehydration, the heads were put into 100% acetone for 30 min and impregnated in a mixture of acetone and DURCUPAN resin (1:1) with gentle rotation overnight. The resin was polymerized by placing the samples in a 60°C oven for 30 min. The heads were sectioned into 60–80 nm slices in an ultramicrotome, and prepared for electron microscopy.[16] Subcellular structures were examined in mosaic and non-mosaic flies, comparing the alterations in mutant with non-mutant ommatidia,[17] and photoreceptor disappearance was also evaluated.

Estimation of lipid peroxidation and apoptosis

An immunohistochemical approach was used to evaluate the onset and progression of lipid peroxidative and apoptotic activities. Peroxidation of lipids was estimated using an antibody against protein adducts with 4-HNE, whereas apoptosis was assessed with an antibody against active caspase 3. Sampling of retinas was carried out every 2–3 d extending 41 (caspase 3) and 50 d (4-HNE) after hatching. The eyes were fixed in a solution of 4% paraformaldehyde, 20% sucrose and sodium phosphate, pH 7.4, for 24 h. Dehydration was made with increasing concentrations of acetone: 30%, 50% and 70%. Retinas were carefully separated from corneas by manipulating a pair of tungsten needles under a stereoscopic microscope.[18] Retinas were blocked with 2% bovine fetal serum, 2% albumin and phosphate buffer containing 0.25% Triton X-100. Incubation with the first antibody (at 1:500 for 4-HNE and 1:25 for caspase 3) was over night at 4°C. The second fluorescein isothiocyanate-conjugated antibody was added at a 1:5000 dilution (for both antibodies) and was incubated for 2 h. After each incubation, the samples were washed three times for 3 min to decrease the non-specific signals. The images were obtained in an epifluorescence microscope and analyzed with Image Pro plus 5.1. The quantitative expression of the lipid peroxidative and apoptotic activities in the pigmented ommatidia and mutant ommatidia of the mosaic eyes involved three steps: (1) calculation of the fluorescent signal detected in the absence of the first antibody; (2) quantification of the signal in the presence of both antibodies; and (3) estimation of the difference between steps 2 and 1 to get the net fluorescent signal. Results were compared with the signal detected in control, non-mosaic flies without pigment.

Even though the caspase 3 antibody was raised from a human epitope, several tests indicated adequate recognition of the *Drosophila* protein[19] (data not shown).

Data analysis

Values are expressed as mean ± standard error of mean. Significance was tested by analysis of variance and the Student's *t*-test. $P < 0.05$ was considered significant.

Results

Time course of electroretinogram disruption

To gain insight into the process of retinal degeneration associated with the absence of IP_3R, electroretinograms were recorded periodically in mosaic flies from day 1 to ≈day 45 after hatching. Representative recordings of pigmented and mutant ommatidia of mosaic flies (5 and 45 d old) are displayed in Figure 1 (panel d). Non-mosaic flies showed normal electroretinograms with well-defined ON and OFF transients and a clear receptor potential (arrows in panels a–c, but recording not shown). The recordings in mosaic flies depicted alterations in both pigmented and mutant ommatidia, with shorter ON and OFF transients (panels a and c) and briefer receptor potential (panel b).

The time course of the electroretinogram disruption in pigmented and mutant ommatidia of mosaic flies showed similar patterns during the first 10 d after hatching. However, a significant shortening in the ON and OFF transients was detected after day 10. In the following days, the OFF transient and the receptor potential became even smaller, resulting in a much distorted electroretinogram by day 30 and thereafter (panels a–d). Electroretinograms performed in pigmented ommatidia of mosaic flies showed smaller, but consistent alterations in the ON and OFF transients and the receptor potential. In contrast, the electroretinograms obtained in control flies expressing IP_3R but without pigment did not show any significant changes from days 1 to 45 after hatching (arrows in panels a–c). Taken together, the electrophysiological results indicate that the lack of IP_3R is compensated during the first 10 d by an unknown adaptive mechanism; however, after ≈day 15 the function of the photoreceptors and their synaptic contacts deteriorate progressively, culminating in a much altered electroretinogram after day 30. The significant alterations in the recordings of the pigmented ommatidia section of the mosaic eye strongly suggest that damage spread from the mutant ommatidia.[12]

Time course of structural modifications of the photoreceptors

The onset and progression of the damage in structural features of pigmented ommatidia and IP_3R-null ommatidia are depicted in Figure 2 and summarized in Table 1. In mosaic eyes, the onset of modified cellular components was detected by day 5, first with subtle alterations even in pigmented ommatidia; in subsequent days the damage in mutant ommatidia became more severe, especially in photoreceptors and endomembranes. Incipient membrane contour alterations was observed in plasma membranes by day 5 (panel b), increasing at day 15 (panel c, white arrows). At this time, sections of some photoreceptors lacking the IP_3R looked deformed and contracted in the rhabdomeric membranes (asterisk in panel c). By day 40, when the degenerative process is more advanced, loss of photoreceptors, broken mitochondria and distorted morphology of the ommatidia were observed clearly. Some changes were also observed in pigmented ommatidia of the mosaic flies, such as contour alterations in membranes

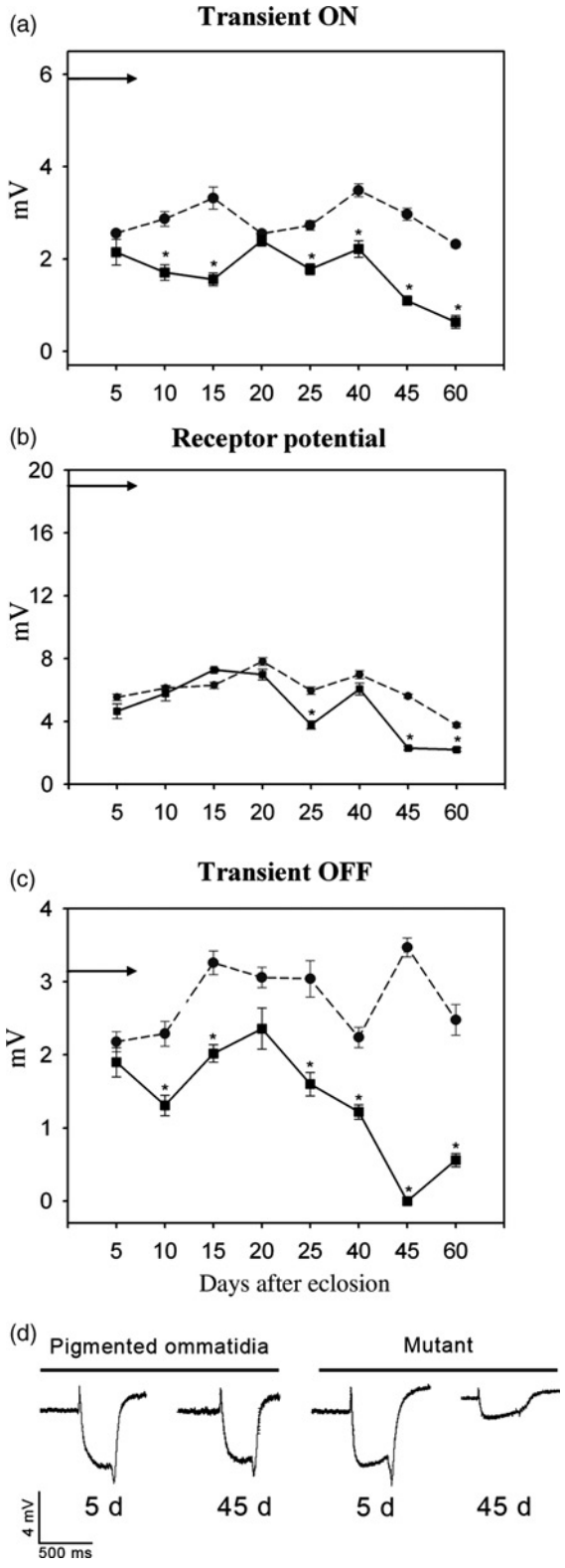

Figure 1 Time course of electroretinogram-related parameters in mosaic flies expressing pigmented and mutant ommatidia. (a–c) Time variations of the ON transient, receptor potential and OFF transient, respectively. Pigmented ommatidia (circles and dash lines) and mutant ommatidia (squares and straight lines). Arrows in panels a–c show the values obtained in recordings of non-mosaic and non-pigmented flies. (d) Representative electroretinograms from mosaic flies from the pigmented ommatidia and mutant zones at 5 and 45 d after eclosion. The values are expressed as mean ± standard error of mean of at least 25 independent observations. *Statistically significant differences, $P < 0.05$

and minor damage in nuclei and mitochondria at day 5, and became more apparent at day 15, probably because of damage spreading from the cells without IP_3R. Some of the alterations observed (membrane contour alterations and broken organells) are characteristics of apoptotic death. In contrast, morphological and ultrastructural characteristics were normal in the non-mosaic control flies (panel a).

Time course of pro-oxidant reactions

Peroxidation of lipids, as an indicator of pro-oxidant reactions, was quantified in *Drosophila* retina *in situ* by an antibody that recognized proteins conjugated with 4-HNE (Figure 3, panel b). Several episodes of enhanced lipid peroxidation were observed beginning in the first days after hatching in the pigmented and mutant ommatidia of mosaic flies, indicating that a burst in oxidant episodes was taking place in ommatidia with and without the expression of the IP_3R (Figure 3, panel a). Five coincident peaks of lipoperoxidative activity were detected in both zones during the course of the experiment (approximately one per week), but the peroxidative bursts expressed by the pigmented ommatidia in the mosaic flies were ≈20% smaller. The signal associated with 4-HNE adducts was barely detectable in non-mosaic control flies (Figure 3, panel a).

Time course of apoptotic activity

Figure 4 (panel a) shows the sequential bursts of activity of the proapoptotic enzyme caspase 3. The activated caspase 3 was detected by immunochemical techniques in the retinas of mosaic flies (Figure 4, panel b). As with the lipid peroxidation results, the pigmented and mutant ommatidia in the mosaic eyes showed coincident increases in apoptotic activity throughout the 40 d of experimental observations. Concurrent increases in caspase 3 signals were detected in five peaks over the course of the 45 d of experimental observations (approximately one per week). Again, fluorescent signal associated with active caspase 3 was barely detectable in the retinas of non-mosaic control flies (Figure 4, panel a).

Discussion

The observation made by Rhagu *et al.*[9] regarding the damage and cellular death in *Drosophila* retinal cells without IP_3R provided an opportunity to examine the relationship between this calcium-release channel and the integrity of specialized cells such as photoreceptors after hatching. *Drosophila* is an accepted experimental model to study degenerative processes since it is possible to study the effects of loss-of-function mutations in mosaic tissues.[12] The use of non-mosaic flies without eye pigment born in the same cohort of mosaics flies demonstrated that the degenerative process observed in mosaic flies was due to the lack of IP_3R and not due to the non-pigmented condition that accompanied construction of the mosaic eyes. In addition, the damage observed in the pigmented

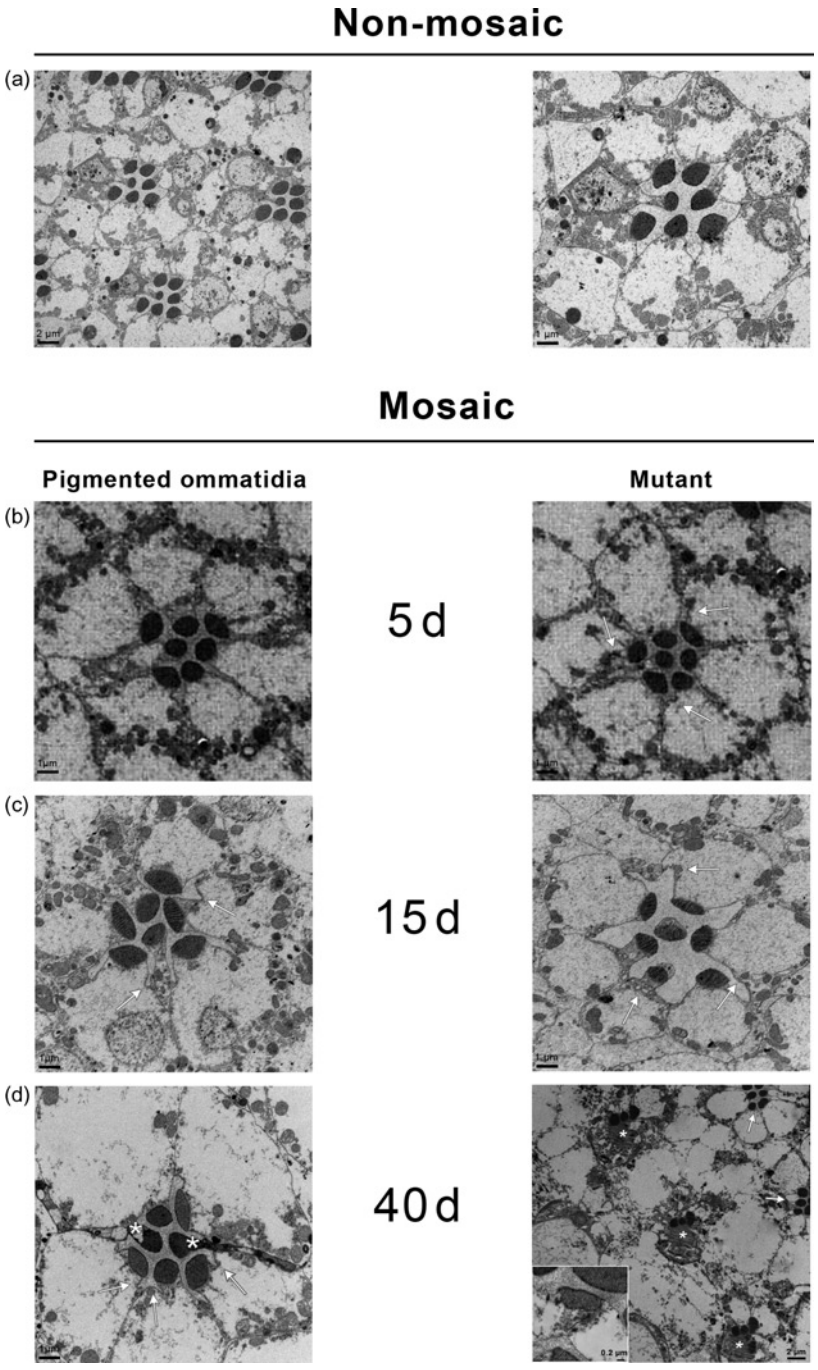

Figure 2 Time course of ultrastructural characteristics in mosaic flies expressing pigmented and mutant ommatidia. (a) Non-mosaic fly expressing IP_3R showing ommatidia with normal cellular structures (left image: low magnification, scale bar = 2 μm; right image: high magnification, scale bar = 1 μm). Panels b–d correspond to mosaic flies: left images show pigmented ommatidia of mosaic flies and right images show mutant ommatidia (scale bar = 1 mm, excepting panel d mutant retina, scale bar = 2 μm). Structural alterations are marked by white arrows in panels b and c; in panel d altered ommatidia are marked with asterisks, whereas normal ommatidia are marked with arrows. Panel d (right) also contains an inset showing a disorganized rhabdomere. Representative images of at least five independent observations

ommatidia of the mosaic flies clearly indicated that the degenerative process was not autonomous, but instead it propagated from the mutant photoreceptors to the rest of the eye cells.[12,13] The exact propagating mechanism for the cellular alterations is not known. Given the plausible pro-oxidant damage in the cells containing the mutant IP_3R, some possibilities might be: (1) the release of ROS/RNA associated with pro-oxidant bursts; (2) the onset of inflammatory reactions promoted by dying cells; and (3) transit of diffusible disability molecules and factors from the affected cells by gap junctions.

Models of retinal degeneration

Numerous mutations expressed in *Drosophila* eye involve some type of retinal degeneration. Alterations in genes of

Table 1 Time course of subcellular alterations detected in mosaic retinas, comparing twin clones of wild-type (W) and mutant (M) ommatidia from mosaics flies

	1 d		5 d		10 d		15 d		30–40 d	
	W	M	W	M	W	M	W	M	W	M
Plasma membrane	*	*	†	‡	†	‡	†	‡	‡	¶
Nuclei	*	*	†	†	†	†	†	†	‡	¶
Mitochondria	*	*	*	†	†	†	†	†	†	‡
Loss of photoreceptors	ND	*	ND	ND	ND	†	ND	†	†	¶
Rhabdomere	*	*	*	*	*	*	*	*	†	†

ND, no detectable damage
Data are derived from at least five independent observations at each time
*Normal
†Mild damage (membrane contour alterations and disorganized rhabdomeres)
‡Medium damage (broken organelles)
¶Severe damage (loss of photoreceptors and ommatidia shrinking and disorganization)

the phototransduction pathway result in morphological abnormalities whose expression is dependent on light stimulation. In contrast, the cellular disruption associated with the absence of IP_3R is light independent.[9] Some other mutations that are also independent of photonic stimulus are: *ninaE* (rhodopsin 1), *ninaA* (cyclophilin-related protein), *rdgA* (diacylglycerol kinase), *arr1* (arrestin 1) and *trp* (transient receptor potential channel).[13] Another aspect that is distinctive about the retinal degeneration in the IP_3R mutant flies is the long time interval before it is established (days to weeks). Retinal degeneration associated with other mutated genes usually exhibit functional or

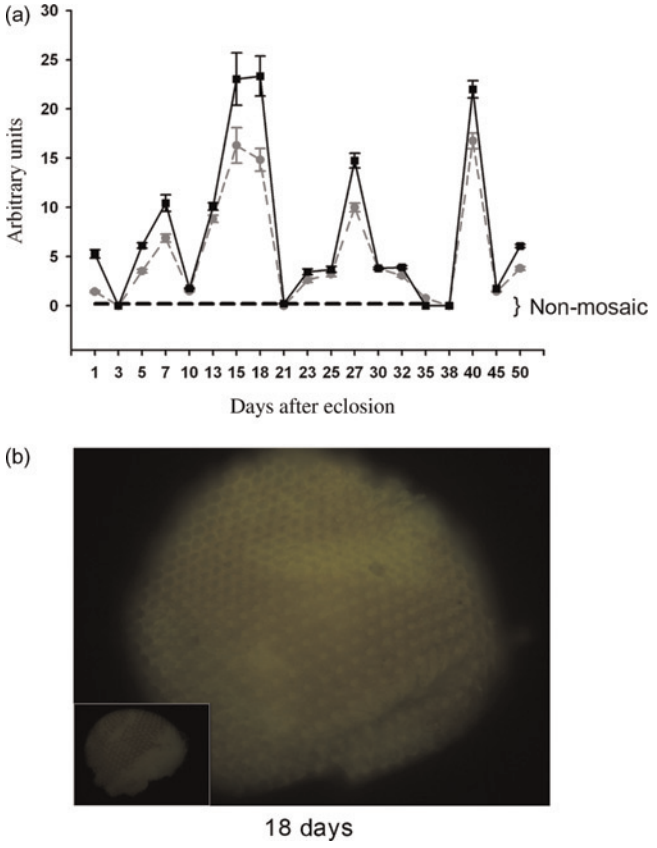

Figure 3 (a) Time course of pro-oxidant reactions detected by fluorescent immunochemical label associated with 4-hydroxy-nonenal (4-HNE) conjugates in mosaic flies lacking IP_3R. Gray circles and dash lines show the signal of pigmented ommatidia, and black squares and straight lines correspond to mutant ommatidia. The thick-black line at the bottom of the graphic shows the lipoperoxidative signal measured in retinas of non-mosaic flies. (b) Representative image showing the fluorescent signal associated with the presence of 4-HNE conjugates from an 18-d- old mosaic fly (the mutant area is non-pigmented). The values are expressed as mean ± standard error of mean of at least 20 independent observations. *Statistically significant differences, $P < 0.05$

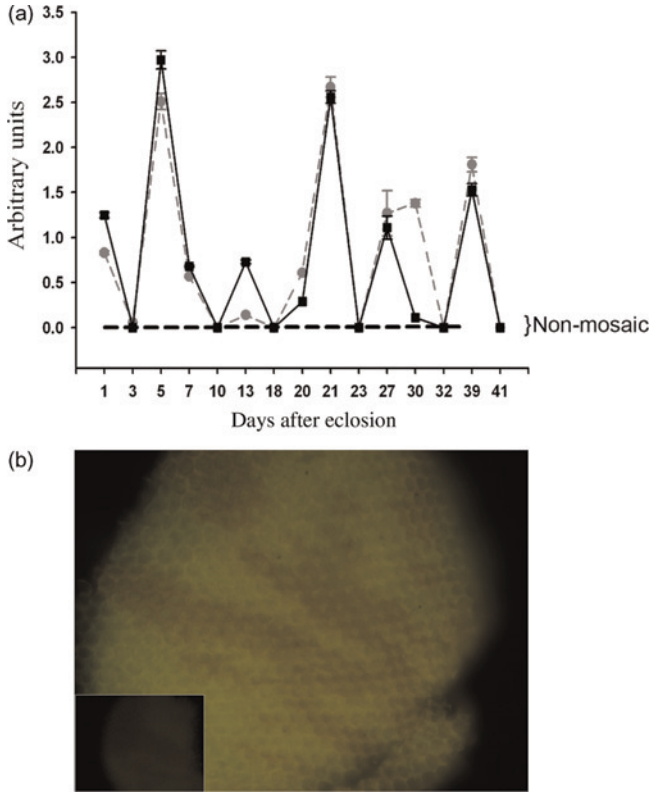

Figure 4 (a) Time course of fluorescent signal associated with active caspase 3 in mosaic flies lacking IP_3R. Gray circles and dash lines show the signal of pigmented ommatidia, and black squares and straight lines correspond to mutant ommatidia. The thick-black line at the bottom of the graphic shows the signal of active caspase 3 measured in retinas of non-mosaic flies. (b) Representative image showing the fluorescent signal associated with the presence of caspase 3 activated from a 21-d-old mosaic fly (the mutant area is non-pigmented). The values are expressed as mean ± standard error of mean of at least 20 independent observations. *Statistically significant differences, $P < 0.05$

morphological abnormalities within only 1 or 2 d (rdgA, rdgB, arr2).[8,20]

IP_3R and calcium mobilization in fly retina

The biophysical properties of the *Drosophila* IP_3R are similar to those reported for its vertebrate counterparts, and more precisely to the IP_3R type 1.[21] The distinctive characteristics of IP_3R type 1 are as follows: medium IP_3 affinity, a high ATP affinity and a low Ca^{2+} affinity.[22] In addition, IP_3R type 1 has been recognized as a critical factor in apoptosis;[1] however, this role has not been studied for the *Drosophila* IP_3R.

As to the role played by the IP_3R in retinal cells, especially in the physiology of photoreceptors, it is accepted that in the same way that occurs in other cellular systems, the IP_3R is localized in endomembranes such as the endoplasmic reticulum and nucleus. Nonetheless, in photoreceptors, the IP_3R fulfills complementary roles, releasing calcium in photon-responsive (submicrovillar cisternae) and in non-responsive zones (perinuclear and cytoplasmic cisternae). It has been suggested that in open-rhabdom eyes such as *Drosophila*'s, the more important calcium reservoir for phototransduction is not the one associated with the submicrovillar cisternae but the extracellular distended lacunae at the bases of the rhabdomeric structures. On the other hand, the rest of the endoplasmic reticulum seems to play a much more important role in the intracellular calcium dynamics by participating in cell signaling, communication with the mitochondrial network, control of pro-oxidant and apoptotic reactions and regulation of protein folding.[23]

In addition to the IP_3R, another calcium-release channel, the RyR, is also present in the *Drosophila* retina.[7] This raises the possibility of a more complex intracellular calcium regulation since it is well documented that when these two receptor channels co-exist in the same cell, they usually act in coordination to fine-tune the mobilization of cytoplasmic and nuclear calcium.[24] RyR is localized in the perinuclear endoplasmic reticulum and in the submicrovillar cisternae, but at a much higher concentration in the former set of endomembranes.[25] Further experiments are needed to find out if the presence and/or properties of the RyR change in the mutant retinal cells lacking the IP_3R.

IP_3R, oxidative stress and cell death

It is well accepted that IP_3R is a modulator of the apoptotic process.[1] Both pro- and antiapoptotic factors are recognized with high affinity by the IP_3R. Knock out of IP_3R genes usually results in the prevention of apoptosis.[26,27] Hence, the degenerative process observed in *Drosophila*'s mosaic eyes in the ommatidia without IP_3R is unexpected.

To suggest a mechanism to explain the cellular events involved in the retinal damage, it is convenient to consider the tight relation between oxidative reactions and intracellular calcium dynamics. Even though the phototransduction process, which depends on extracellular calcium, is not affected by the lack of IP_3R, it is necessary to characterize the cellular events within the photoreceptors that are regulated by the calcium released from intracellular deposits.

Temporal integration

Our study detected two phases in the degenerative process associated with the lack of IP_3R in the retina: the first encompasses the first 10–15 d after eclosion, when the structural and functional damage are mild. In contrast, the second phase, from day 15 and on, involves severe abnormalities that compromise the integrity of the photoreceptor.

Phase 1: The mutant flies lacking retinal IP_3R showed distorted electroretinograms 10 d after hatching, indicating an altered membrane potential and synaptic activity (Figure 1). The first defects, the loss of the ON and OFF transients, are usually associated with the impaired electrical communication in the first synapse of the visual pathway between the photoreceptor and the neurons at the lamina layer. On the other hand, structural abnormalities in the mutant retinas started at day 5, with incipient membrane contour alterations in the photoreceptor membrane that became more evident by days 10–15 (Figure 2). Remarkably, increased episodes of lipoperoxidative and caspase 3 activities were detected during the first week after eclosion, suggesting that functional and structural defects were preceded by enhanced pro-oxidant reactions as well as an activated program of cell death (Figures 3 and 4).

Phase 2: Electroretinograms and membrane structures were seriously modified: the electrical response to photonic stimulation is almost lost, and the photoreceptor morphology is highly disorganized, even showing loss of rhabdomeric structures. The increased lipid peroxidation and the caspase 3 activity continue throughout the entire second phase (Figures 3 and 4). One interpretation of these data is that, in the absence of IP_3R, the system reaches a point at which it is no longer able to sustain equilibrium between pro-oxidant reactions and antioxidant defenses. Beyond that point ($\approx$day 15 after hatching), the degenerative process becomes irreversible.

In summary, our results indicate that the absence of IP_3R in the retina promotes continuous and propagating episodes of oxidative stress and apoptotic activity during the entire post-hatching period. These intermittent episodes of pro-oxidant and apoptotic reactions have been observed in other experimental systems such as liver regeneration and it has been explained as events of cellular damage promoted by alteration in redox balance.[28] During the first 10 d the system can compensate, although not completely, for the increase in pro-oxidant reactions and the enhanced apoptotic activity. The compensatory mechanisms might involve antioxidant defenses and increased cellular exchange. However, after 15 d the system loses its capacity to compensate for or repair the oxidative damage, and a cascade of deleterious effects make the retinal cells unable to survive. It would be opportune to explore if necrotic events also take place along with apoptosis during the degenerative process promoted by the null IP_3R mutation in *Drosophila*.

Author contributions: OV-M performed all the experiments and participated in drafting the manuscript; AL participated in generating the mosaics and implemented the technique to obtained the retinas and the immunohistochemical experiments; LP-T helped with the electron microscopy; SW-F

helped with the quantification of fluorescent signals; MV-L helped in obtaining the mosaic flies, isolating retinas and with the oxidative stress experiments; AA helped in the mating of flies to get the mosaic stocks; JR-E contributed the fly stocks, helped with the genetic experiments and participated in drafting the manuscript; RH-M participated in drafting the manuscript; and MD-M conceived the study, participated in designing the project and drafting the manuscript.

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REFERENCES

1 Choe CU, Ehrlich BE. The inositol 1, 4, 5-trisphosphate receptor (IP_3R) and its regulators: sometimes good and sometimes bad teamwork. *Sci STKE* 2006;**363**:re15
2 Clapham DE. Calcium signaling. *Cell* 2007;**131**:1047–58
3 Verkhratsky A. Calcium and cell death. *Subcell Biochem* 2007;**45**:465–80
4 Bezprozvanny I. Inositol 1, 4, 5-trisphosphate receptor, calcium signaling and Huntington's disease. *Subcell Biochem* 2007;**45**:323–35
5 Venkatesh K, Hasan G. Disruption of the IP_3 receptor gene of *Drosophila* affects larval metamorphosis and ecdysone release. *Curr Biol* 1997;**7**:500–9
6 Yoshikawa S, Tanimura T, Miyawaki A, Makoto N, Yusaki M, Furuichi T, Mokoshiba K. Molecular cloning and characterization of the inositol 1, 4, 5-trisphosphate receptor in *Drosophila melanogaster*. *J Biol Chem* 1992;**267**:16613–9
7 Vázquez-Martínez O, Cañedo-Merino R, Díaz-Muñoz M, Riesgo-Escovar JR. Biochemical characterization, distribution and phylogenetic analysis of *Drosophila melanogaster* ryanodine and IP_3 receptors, and thapsigargin-sensitive Ca^{2+} ATPase. *J Cell Sci* 2003;**116**:2483–94
8 Hardie RC. Phototransduction in microvillar photoreceptors of *Drosophila* and other invertebrates. In: Basbaum AI, Kaneko A, Shepherd GM, Westheimer G, eds. *The Senses: A Comprehensive Reference, Vision 1*. Vol. 1. San Diego: Academic Press, 2008
9 Raghu P, Colley NJ, Webel R, James T, Hasan G, Danin M, Selinger Z, Hardie RC. Normal phototransduction in *Drosophila* photoreceptors lacking an $InsP_3$ receptor gene. *Mol Cell Neurosci* 2000;**15**:429–45
10 Acharya JK, Jalink K, Hardy RW, Hartenstein V, Zuker CS. $InsP_3$ receptor is essential for growth and differentiation but not for vision in *Drosophila*. *Neuron* 1997;**18**:881–7
11 Dröge W. Free radicals in the physiological control of cell function. *Physiol Rev* 2002;**82**:47–95
12 Resino J, García-Bellido A. *Drosophila* genetic variants that change cell size and rate of proliferation affect cell communication and hence patterning. *Mech Develop* 2004;**121**:351–64
13 Theodosiou NA, Xu T. Use of FLP/FRT system to study *Drosophila* development: a companion to methods. *Enzymology* 1998;**14**:355–65
14 Perrimon N. Creating mosaics in *Drosophila*. *Int J Dev Biol* 1998;**42**:243–7
15 Alawi AA, Pak WL. On-transient of insect electroretinogram: its cellular origin. *Science* 1971;**172**:1055–7
16 Cárabez-Trejo A, Sandoval F, Palma-Tirado L. Ultrastructural changes of tissues produced by inhalation of thinner in rats. *Microsc Res Technol* 1998;**40**:56–62
17 Bursch W, Ellinger A, Gerner C, Fröhwein U, Schulte-Hermann R. Programmed cell death (PCD) apoptosis, autophagic PCD, or others? *Ann NY Acad Sci* 2000;**926**:1–12
18 Hiromi Y. Whole mount staining of *Drosophila* retina for β-galactosidase activity. In: *Rubin Lab Methods*. Berkeley, CA, 1990:78
19 Ohsawa S, Hamada S, Yoshida H, Miura M. Caspase-mediated changes in histone H1 in early apoptosis: prolonged caspase activation in developing olfactory sensory neurons. *Cell Death Differ* 2008;**15**:1429–39
20 Acharya U, Patel S, Koundakjian E, Nagashima K, Han X, Acharya JK. Modulating sphingolipid biosynthetic pathway rescues photoreceptor degeneration. *Science* 2003;**14**:1740–3
21 Srikanth S, Wang Z, Tu H, Nair S, Mathew MK, Hasan G, Bezprozvanny I. Functional properties of the *Drosophila melanogaster* inositol 1, 4, 5-trisphosphate receptor mutants. *Biophys J* 2004;**86**:3634–46
22 Tu H, Wang Z, Nosyreva E, De Smedt H, Bezprozvanny I. Functional characterization of mammalian inositol 1, 4, 5-trisphosphate receptor isoforms. *Biophys J* 2005;**88**:1046–55
23 Polyanovsky AD. Calcium stores in *Drosophila* retina revisited: an electron–cytochemical localization of calcium. *J Evol Biochem Physiol* 2000;**36**:78–84
24 Morales-Tlalpan V, Arellano RO, Díaz-Muñoz M. Interplay between ryanodine and IP_3 receptors in ATP-stimulated mouse luteinized-granulosa cells. *Cell Calcium* 2005;**37**:203–13
25 Baumann O. Distribution of ryanodine receptor Ca^{2+} channels in insect photoreceptors cells. *J Comp Neurol* 2000;**421**:347–61
26 Hirota J, Baba M, Matsumoto M, Furuichi T, Takatsu K, Mikoshiba K. T-cell-receptor signaling in inositol 1, 4, 5-trisphosphate receptor (IP_3R) type-1-deficient mice: is IP_3R type 1 essential for T-cell-receptor signaling? *Biochem J* 1998;**333**:615–9
27 Jayaraman T, Marks A. T cells deficient in inositol 1, 4, 5-trisphosphate receptor are resistant to apoptosis. *Mol Cell Biol* 1997;**17**:3005–12
28 Aguilar-Delfín I, López-Barrera F, Hernández-Muñoz R. Selective enhancement of lipid peroxidation in plasma membrane in two experimental models of liver regeneration: partial hepatectomy and acute CC14 administration. *Hepatology* 1996;**24**:657–62

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