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Ecdysone-dependent and ecdysone-independent programmed cell death in the developing optic lobe of *Drosophila*

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ABSTRACT

The adult optic lobe of *Drosophila* develops from the primordium during metamorphosis from mid-3rd larval stage to adult. Many cells die during development of the optic lobe with a peak of the number of dying cells at 24 h after puparium formation (h APF). Dying cells were observed in spatio-temporal specific clusters. Here, we analyzed the function of a component of the insect steroid hormone receptor, EcR, in this cell death. We examined expression patterns of two EcR isoforms, EcR-A and EcR-B1, in the optic lobe. Expression of each isoform altered during development in isoform-specific manner. EcR-B1 was not expressed in optic lobe neurons from 0 to 6 h APF, but was expressed between 9 and 48 h APF and then disappeared by 60 h APF. In each cortex, its expression was stronger in older glia-ensheathed neurons than in younger ones. EcR-B1 was also expressed in some types of glia. EcR-A was expressed in optic lobe neurons and many types of glia from 0 to 60 h APF in a different pattern from EcR-B1. Then, we genetically analyzed EcR function in the optic lobe cell death. At 0 h APF, the optic lobe cell death was independent of any EcR isoforms. In contrast, EcR-B1 was required for most optic lobe cell death after 24 h APF. It was suggested that cell death cell-autonomously required EcR-B1 expressed after puparium formation. βFTZ-F1 was also involved in cell death in many dying-cell clusters, but not in some of them at 24 h APF. Altogether, the optic lobe cell death occurred in ecdysone-independent manner at prepupal stage and ecdysone-dependent manner after 24 h APF. The acquisition of ecdysone-dependence was not directly correlated with the initiation or increase of EcR-B1 expression.
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Introduction

During development of the central nervous system, excess neurons are initially produced, then superfluous neurons die, and surviving neurons form mature neural circuits. To reveal the mechanism and the significance of this cell death is essential for understanding neural development. In the *Drosophila* optic lobe, many cells die during development (Fischbach and Technau, 1984; Hofbauer and Campos-Ortega, 1990; Togane et al., 2012). Here, we investigate the role of the insect steroid hormone, ecdysone, in this cell death.

The optic lobe processes and transmits visual information from the compound eye to the central brain. It consists of four neuropils—the lamina, medulla, lobula, and lobula plate—and surrounding cortices. During metamorphosis, the optic lobe develops from the optic lobe primordium, which includes two proliferation centers—the outer optic anlagen (OOA) and the inner optic anlagen (IOA) (Fischbach and Hiesinger, 2008; Meinertzhagen and Hanson, 1993). Lamina neurons are produced by precursor cells that receive signals from photoreceptor axons on the lateral side of the OOA (Huang and Kunes, 1998, 1996; Huang et al., 1998; Selleck et al., 1992; Selleck and Steller, 1991; Umetsu et al., 2006). Medulla neurons differentiate from neuroblasts that are produced on the medial side of the OOA under the control of several signaling pathways (Egger et al., 2010; Fischbach and Hiesinger, 2008; Meinertzhagen and Hanson, 1993; Ngo et al., 2010; Wang et al., 2011a, 2011b; Yasugi et al., 2010, 2008). T2/T3/C neurons and lobula plate neurons are derived from the IOA; however, the development of these neurons has not been described in detail. The optic lobe glia also develop during optic lobe development, and they have essential roles in the formation of neural circuits and neuropil boundaries, proliferation and survival of neurons, and functions of synapses (reviewed by Chotard and Salecker, 2007; Edwards and Meinertzhagen, 2010).

Cell death in the optic lobe occurs in a specific spatio-temporal pattern (Togane et al., 2012). It occurs in two rounds. The first round begins at the onset of metamorphosis and continues until 48 h APF. Many cells die in this round, with a peak at 24 h APF. Dying cells are distributed in specific regions of the cortices, and are evident as distinct clusters. These clusters change in size, density, and position as development proceeds. Most dying cells are neurons, and the others are glia. The second round of cell death occurs from 48 h APF

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to eclosion; only a few cells die during this second round. The optic lobe cell death is apoptosis (Togane et al., 2012).

Ecdysone is an insect steroid hormone that regulates larval development and metamorphosis. During the first half of metamorphosis, when the first round of cell death takes place, there are three ecdysone pulses—the late-larval pulse, the prepupal pulse, and the pupal pulse (Riddiford, 1993). The late-larval pulse peaks at the onset of metamorphosis and initiates pupariation. The prepupal pulse is a small peak at 10 h APF and is necessary for the prepupal–pupal transition. The pupal pulse is the large surge that peaks about 30 h APF and is responsible for adult development.

Ecdysone is received by a heterodimeric nuclear hormone receptor, EcR/USP (Koelle et al., 1991; Riddiford et al., 2000; Thomas et al., 1993; Yao et al., 1993, 1992). The receptor binds to ecdysone response elements (EcREs) to regulate target gene expression in multiple tissues (Cherbas et al., 1991; Riddihough and Pelham, 1987). The *EcR* gene encodes three isoforms—EcR-A, EcR-B1, and EcR-B2 (Talbot et al., 1993). These isoforms result from alternative splicing, and all share a common DNA binding domain and a common ligand binding domain, but each has a unique A/B domain in their N-terminal. EcR-B1 and EcR-B2 each have activation function (AF1) in the A/B domain; EcR-A lacks AF1 and instead has an inhibitory function in the A/B domain; EcR-B1 and EcR-B2 lack this inhibitory function (Mouillet et al., 2001). EcR-A and EcR-B1 isoforms are expressed in different spatial and temporal patterns, and are suggested to have different functions (Talbot et al., 1993; Truman et al., 1994). In fact, loss of EcR-B1 leads to developmental arrest at the onset of metamorphosis and defects in many developmental events—including a pruning of neurons, tanning of puparium, and replacement of larval with imaginal tissues in the midgut and abdomen (Bender et al., 1997; Lee et al., 2000). In contrast, EcR-A mutants arrest development at mid- and late-pupal stages and have abnormally persistent salivary glands and malformed legs (Davis et al., 2005).

Many researchers have reported that ecdysone-regulated cell death occurs during metamorphosis (reviewed by Yin and Thummel, 2005). In the first half of metamorphosis, ecdysone activates cell death in the larval midgut (Jiang et al., 1997; Lee et al., 2002a), salivary glands (Jiang et al., 1997, 2000; Lee et al., 2002b), and two distinct groups of neurons in the ventral nervous system—vCrz neurons (Choi et al., 2006) and RP2 neurons (Winbush and Weeks, 2011). The vCrz neurons require EcR-B2 for cell death; in contrast, the RP2 neurons require EcR-B (B1 and/or B2). Moreover, there is molecular genetic relationship between ecdysone signals and cell death; specifically, *reaper*, a cell death-inducing factor, and *Dronc*, an initiator caspase, each have an EcRE within their respective promoters (Cakouros et al., 2004; Jiang et al., 2000).

In this study, we analyzed the role of ecdysone signaling in the cell death that occurs during development of the optic lobe; specifically, we focused on the expression and function of EcR-A and EcR-B1. First, we examined expression of each isoform and found that they were expressed in different patterns both in neurons and in glia within the developing optic lobe. We then used isoform-specific mutants to investigate the function of each protein and found that cell death in the prepupal stage occurred in an ecdysone-independent manner and the cell death in the later stages occurred in an ecdysone-dependent manner. Moreover, we found that the ecdysone-dependent cell death required EcR-B1.

Materials and methods

Fly strains

Canton-Special was used as the wild-type strain. We used three GAL4 lines, *M1B repo-GAL4* (Sepp et al., 2001), *C155 elav-GAL4* (Lin and Goodman, 1994) and *NP6099-GAL4* (Hayashi et al., 2002), and three UAS lines, *UAS-GFP.nls14* (Bloomington *Drosophila* Stock Center), *UAS-P35.H* (Bloomington *Drosophila* Stock center) and *UAS-EcR-RNAi*^{104} (Colombani et al., 2005). We used two EcR-A mutants, *EcR*^{112} and *EcR*^{139} (Carney et al., 2004), and two EcR-B1 mutants, *EcR*^{Q50st} and *EcR*^{W53st} (Bender et al., 1997). The constructs used to knockdown EcR and FTZ-F1 were *hs-EcRi-11* and *hs-FFi-24*, respectively (Lam and Thummel, 2000). Flies were reared on a general cornmeal-yeast medium at 25 °C under 12 h/12 h light/dark photoperiod.

Detection of dying cells

A modified version of the TUNEL (terminal deoxynucleotide transferase dUTP nick and labeling) method described by Kimura (1995) was used to detect dying cells. Briefly, brains were excised, washed in PBS, fixed in 4% formaldehyde, washed with PBS containing Triton-X (PBS-Tx), and stored in methanol at −20 °C for at least one night. These brains were then washed with PBS-Tx, treated with Protease K (Wako Pure Chemical Industries, Osaka) for 10 min at room temperature, and fixed again in 4% formaldehyde. The brains were subsequently washed with PBS-Tx, pretreated in terminal deoxynucleotidyl transferase (TdT) buffer, and incubated overnight at 37 °C in TdT reaction solution (Takara Bio, Shiga) with biotin-16-dUTP (Roche Diagnostics, Mannheim). The brains were next washed with PBS-Tx and incubated in ABC reaction solution (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. After washing with PBS-Tx, the brains were stained using a standard brown horseradish peroxidase reaction with 3,3′-diaminobenzidine and hydrogen peroxide. Dying cells appeared dark brown. After the reaction, the brains were washed with distilled water, dehydrated in a series of ethanol solutions of increasing concentrations, cleared with methyl benzoate, and embedded in Canada Balsam:methyl benzoate 3:1 (V:V) on a glass slide. The specimens were inspected with bright-field microscopy (Optiphot, Nikon, Tokyo).

Analysis of the number and distribution of dying cells

We used a light microscope, a × 40 objective lens, and a digital camera (TS-CA series, Sugitoh Co., Ltd., Tokyo) to acquire images of dying cells; we then digitally enlarged these images to × 1000 on a monitor. While we gradually shifted the focus, each signal larger than 0.7 μm in diameter was scored as a dying cell with a dot on a transparent plastic sheet that was affixed to the monitor. We used images from the anterior view to count the number of dying cells. The images were captured digitally with a scanner and the number of dying cells was counted using the particle counter module of the NIH ImageJ software package. For the analysis of spatial distribution of dying cells, we obtained images of 1 μm-thick serial optical sections that were taken from a dorsal view. Optical microscope (BX50, Olympus, Tokyo), a digital camera (DP72, Olympus, Tokyo), and Metamorph software (MDS Analytical Technologies, Sunnyvale) were used for this imaging.

Heat induction of dsRNA

To induce RNAi with EcR and βFTZ-F1 before puparium formation, late third instar larvae were placed into 1.5 ml microcentrifuge tubes, and these tubes were incubated for 30 min in a 37 °C water bath. Then, the larvae were transferred into new culture vials and maintained at 25 °C. The animals that underwent pupariation 12–16 h after the heat-shock were used for the experiments. To induce RNAi with EcR after puparium formation, prepupae were subjected to a heat shock twice. They were placed into 1.5-ml microcentrifuge tubes at 0 h APF, and these tubes

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