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Fates of identified pioneer cells in the developing antennal nervous system of the grasshopper *Schistocerca gregaria*

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ABSTRACT

In the early embryonic grasshopper, two pairs of sibling cells near the apex of the antenna pioneer its dorsal and ventral nerve tracts to the brain. En route, the growth cones of these pioneers contact a so-called base pioneer associated with each tract and which acts as a guidepost cell. Both apical and basal pioneers express stereotypic molecular labels allowing them to be uniquely identified. Although their developmental origins are largely understood, the fates of the respective pioneers remain unclear. We therefore employed the established cell death markers acridine orange and TUNEL to determine whether the apical and basal pioneers undergo apoptosis during embryogenesis. Our data reveal that the apical pioneers maintain a consistent molecular profile from their birth up to mid-embryogenesis, at which point the initial antennal nerve tracts to the brain have been established. Shortly after this the apical pioneers undergo apoptosis. Death occurs at a developmental stage similar to that reported elsewhere for pioneers in a leg – an homologous appendage. Base pioneers, by contrast, progressively change their molecular profile and can no longer be unequivocally identified after mid-embryogenesis. At no stage up to then do they exhibit death labels. If they persist, the base pioneers must be assumed to adopt a new role in the developing antennal nervous system.

1. Introduction

Pioneer neurons are cells whose axons first navigate a pathway in the developing nervous system and have been intensively studied in both vertebrates and invertebrates (Ramón y Cajal, 1890; McConnell et al., 1989; Supèr et al., 1998; Whitlock and Westerfield, 1998; Bate, 1976; Miguel-Allaga and Thor, 2004; Raper et al., 1983; Goodman and Doe, 1994; Boyan et al., 1995; Ganfornina et al., 1995; Sánchez et al., 1995). The insect appendages, in particular, have proven to be an advantageous model system for studying the cellular and molecular aspects of pioneer cell biology (Bate, 1976; Keshishian, 1980; Bentley and Keshishian, 1982; Ho and Goodman, 1982; Meier and Reichert, 1991; Bentley and O'Connor, 1992; Seidel and Bicker, 2000). The grasshopper antenna is such an appendage, comprising scape, pedicel and flagellum (Chapman, 1982). Externally, the flagellum is characterized by segments called meristal annuli (Chapman, 2002), whose borders are delimited early in embryogenesis by epithelial bands of Annulin (Bastiani et al., 1992), Lazarillo (Boyan and Williams, 2004), and Lachesin (Ehrhardt et al., 2015a) labeling. The early embryonic antenna comprises four such annuli termed A1, A2, A3, A4 starting from the apex. These meristal segments multiply during subsequent embryonic and post-embryonic development so that the flagellum lengthens (Chapman, 2002). Ontogenetically, the antenna comprises an outer epithelium of ectodermal origin from which sensory cells differentiate (Chapman and Greenwood, 1986; Ochieng et al., 1998), and an inner lumen of mesodermal origin containing the median strand, coelom, trachea, hemocytes and nerve tracts (see Gewecke, 1972; Anderson, 1973; Chapman, 1982).

The grasshopper antenna has two nerve tracts, one dorsal and one ventral, each established during early embryogenesis by its own set of pioneers which delaminate from the epithelium into the lumen at the antennal tip and direct axons towards the brain (Bate, 1976; Ho and Goodman, 1982). These apical pioneers can be uniquely identified via the neuron-specific label horseradish peroxidase (HRP, see Jan and Jan, 1982; Boyan and Williams, 2004; Seidel and Bicker, 2000) and the cell surface GPI-linked lipocalin Lazarillo (Ganfornina et al., 1995; Boyan and Williams, 2004). Near

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the base of the antenna, the growth cones of sibling apical pioneers from each tract encounter a guidepost-like cell called a base pioneer (Ho and Goodman, 1982; Berlot and Goodman, 1984; Seidel and Bicker, 2000), one of which is now known to be associated with each pathway (Ehrhardt et al., 2015a). Antibody blocking of Lazarillo, which is expressed by both the apical pioneers and their target base pioneers, has been shown to disrupt axogenesis in this pathway (Ehrhardt et al., 2015a) as it does in the grasshopper central nervous system (Sánchez et al., 1995).

Despite considerable knowledge about the origin of these pioneers (Ho and Goodman, 1982; Seidel and Bicker, 2000; Ehrhardt et al., 2015a), their fate is unknown. Pioneers of the peripheral nervous system of the leg die shortly after mid-embryogenesis (Kutsch and Bentley, 1987). Insect appendages are serially homologous (Gibson and Gehring, 1988; Casares and Mann, 1998), and their nervous systems develop along homologous lines (Meier and Reichert, 1991). It is therefore conceivable that the pioneers of both the antennal and leg nervous systems suffer the same fate. This possibility represents the focus of our study.

Our findings show that while the apical pioneers are indeed labeled by molecular markers associated with programmed cell death at the equivalent age to those in the leg, the base pioneers are not labeled even at mid-embryogenesis. Instead, the base pioneers progressively downregulate their HRP- and Lazarillo-expression, thereby altering their molecular profile. We speculate that these findings indicate different fates for the apical and base pioneers in the developing antennal nervous system.

2. Materials and methods

2.1. Animals and preparation

Eggs were produced by a crowded colony of *Schistocerca gregaria* with a 12/12 h light/dark regime, 35% air humidity, a day temperature of 30 °C and a night temperature of 20 °C. Eggs were incubated in moist, aerated containers under this same regime. Embryos were staged according to Bentley et al. (1979). The staging protocol becomes less accurate with age, so that at mid-embryogenesis an uncertainty of ±1% is not uncommon over 20 days of embryogenesis. Staged embryos were removed from the egg and embryonic membranes in 0.1 M phosphate buffed saline (PBS: 2 mM NaH_2PO_4 monohydrate; 16 mM Na_2HPO_4 anhydrous; 175 mM NaCl, adjusted to pH 7.4 with NaOH).

2.2. Apoptosis labels

2.2.1. Acridine orange (AO)

This green fluorescent dye (3,6-bis(dimethylamino) acridine; Sigma-Aldrich, 235474) is a vital stain which binds to nucleic acids (Söderström et al., 1977). Acridine orange has previously been used to detect programmed cell death during insect development (Spreij, 1971; Abrams et al., 1993; Jiang et al., 1997; Liu and Boyan, 2013; Boyan and Liu, 2014). Apoptotic cells are selectively labeled by AO and the dye does not mark necrotically dying cells (Abrams et al., 1993). After removal from the egg, wholemount preparations were incubated in 5 μg/ml AO in PBS for 30 min at room temperature on a shaker.

2.2.2. TUNEL

This method (terminal deoxynucleotidyl transferase deoxyuridine triphosphate nucleotides (dUTP) nick end labeling) detects the fragmentation of DNA which occurs during apoptosis (Gavrieli et al., 1992). TUNEL has been used to investigate the DNA fragmentation accompanying programmed cell death during development in insects (Jiang et al., 1997; Bello et al., 2003; Lobbia et al., 2003; Page and Olofsson, 2008; Boyan and Liu, 2014). After being fixed in 3.7% PFA (paraformaldehyde in PBS) overnight, embryos were permeabilized to enable TUNEL labeling. Embryos were washed four times (10 min each) in PBT (0.1 M PBS with 0.1% Triton-X, Sigma), then placed in an Eppendorf tube with 500 μl of fresh 0.1% PBT. Embryos at 55% or older were sonicated with a Bandelin SONOREX™ rk100 ultrasound (35 kHz) bath sonicator; younger embryos were sonicated at half power (see Ehrhardt et al., 2015b). Embryos were then placed in 0.1 M citrate buffer (19 mM citric acid; 81 mM sodium citrate, adjusted to pH 6.0 with NaOH), and placed in a microwave oven for 5 min at 360 W in order to permeabilize the tissue. The embryos were then washed overnight in PBS at 4 °C. A preincubation medium of 20% normal goat serum (NGS) and 3% bovine serum albumin (BSA) in 0.1 M TrisHCl (0.1 M Tris in distilled water, adjusted to pH 7.5 with HCl) was applied for 30 min at room temperature to denature the DNA. Embryos were washed three times (10 min each) in 0.1 M PBS at room temperature. The preparations were then labeled using the *In Situ* Cell Death Detection Kit, TMR red (Roche) for 2 h at 37 °C. They were washed in 0.1 M PBS overnight before HRP immunolabeling.

2.3. Immunolabeling: primary antibodies

2.3.1. Horseradish peroxidase (HRP)

The antibody against HRP is used as a specific neuronal marker in insects (Jan and Jan, 1982). After fixation in 3.7% PFA overnight at 4 °C, preparations were washed in PBS for 6 h. For anti-HRP labeling on wholemount preparations, a polyclonal anti-HRP antibody in rabbit (Dianova, 323-005-021) was diluted 1:200 in preincubation medium (0.1 M PBS, 0.5% Triton-X, 1% NGS, 3% BSA). Preparations were incubated in the medium for 3 days at 4 °C in the dark. For HRP labeling on sections, an anti-HRP antibody in goat (Dianova, 123-005-021) was diluted 1:200 in preincubation medium (0.1 M PBS, 0.5% Triton-X, 1% Normal Horse Serum (NHS), 3% BSA). Sections were placed on slides and incubated in the medium for 24 h at 4 °C in the dark.

2.3.2. Mes3

The anti-Mes3 antibody binds to a cell surface epitope expressed by cells of mesodermal origin (Kotrla and Goodman, 1984; Boyan and Williams, 2007; Ehrhardt et al., 2015a). Prior to Mes3 immunolabeling, wholemount embryos were fixed in PIPES-FA (3.7% paraformaldehyde in 100 mM PIPES, 2 mM EGTA, 1 mM $MgSO_4$) for 1 h at room temperature, then washed in PBS for 1 h. Embryos were preincubated in PBS with 0.1% Triton-X and 5% fetal calf serum for 1 h at room temperature. The Mes3 antibody raised in mouse (gift of C. Goodman) was diluted 1:4 in the preincubation medium, along with the anti-HRP antibody in rabbit diluted 1:200. Embryos were incubated in the antibodies for 3 days at 4 °C in the dark.

2.4. Secondary antibodies

Following incubation in the primary antibody, preparations were washed six times in 0.1 M PBS (3 h total washing time) before being placed in an incubation medium containing the secondary antibody. For HRP single labeling of wholemount embryos, and for HRP labeling in combination with TUNEL, the secondary antibody was goat anti-rabbit Alexa 488 (Invitrogen, A11034) diluted 1:250 in 0.1 M PBS, 0.05% Triton-X, 1% NGS and 3% BSA. When HRP immunolabeling was used in combination with AO the secondary antibody against HRP was instead goat anti-rabbit Cy3 (Dianova, 111-165-003), diluted 1:200 in the same medium as above. For HRP single labeling of sections, the secondary antibody was donkey anti-goat Cy3 diluted 1:200 in 0.1 M PBS, 0.05% Triton-X, 1% NHS,