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Hydroxyurea-mediated neuroblast ablation establishes birth dates of secondary lineages and addresses neuronal interactions in the developing *Drosophila* brain

Jennifer K. Lovick, Volker Hartenstein *

Department of Molecular Cell and Developmental Biology, University of California, Los Angeles, Los Angeles, CA 90095, USA

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ABSTRACT

The *Drosophila* brain is comprised of neurons formed by approximately 100 lineages, each of which is derived from a stereotyped, asymmetrically dividing neuroblast. Lineages serve as structural and developmental units of *Drosophila* brain anatomy and reconstruction of lineage projection patterns represents a suitable map of *Drosophila* brain circuitry at the level of neuron populations ("macro-circuitry"). Two phases of neuroblast proliferation, the first in the embryo and the second during the larval phase (following a period of mitotic quiescence), produce primary and secondary lineages, respectively. Using temporally controlled pulses of hydroxyurea (HU) to ablate neuroblasts and their corresponding secondary lineages during the larval phase, we analyzed the effect on development of primary and secondary lineages in the late larval and adult brain. Our findings indicate that timing of neuroblast re-activation is highly stereotyped, allowing us to establish "birth dates" for all secondary lineages. Furthermore, our results demonstrate that, whereas the trajectory and projection pattern of primary and secondary lineages is established in a largely independent manner, the final branching pattern of secondary neurons is dependent upon the presence of appropriate neuronal targets. Taken together, our data provide new insights into the degree of neuronal plasticity during *Drosophila* brain development.

Introduction

The *Drosophila* brain develops from a stereotyped set of embryonically-born stem cells, called neuroblasts. Each neuroblast is defined by its expression of a unique combination of transcriptional regulators (Skeath and Thor, 2003; Urbach and Technau, 2003b). Neuroblasts divide asymmetrically, each mitotic division resulting in a self-renewing neuroblast and a "ganglion mother cell," which divides once more giving rise to two postmitotic neurons. In holometabolous insects, such as *Drosophila*, neuroblasts undergo two phases of proliferation. The first phase occurs during the embryonic period; the second one takes place in the larva. In the embryo, a neuroblast divides five to eight times, producing groups ("lineages") of 10–20 embryonic ("primary") neurons each (Larsen et al., 2009). Neurons belonging to the same lineage share a number of fundamental morphological characteristics: cell bodies remain clustered together in the outer layer (cortex) of the brain and their axons fasciculate into a common tract (primary axon tract; PAT). In cases where clones of differentiated primary neurons have been labeled it became apparent that neurons of one lineage also share one or a few specific brain compartments in which they form synaptic contacts. For example, four lineages (MB1–4) are restricted to the calyx and lobes of the mushroom body (Ito et al., 1997) and one lineage (BAmv3) forms the projection neurons of the larval antennal lobe (Das et al., 2013; Python and Stocker, 2002; Ramaekers et al., 2005).

At the end of embryogenesis, most neuroblasts enter a period of quiescence. Only five neuroblasts (MB1–4, BAlc/LNb) continuously divide between embryogenesis and early metamorphosis (Ito and Hotta, 1992; Ito et al., 1997; Stocker et al., 1997). All other neuroblasts exit the quiescent phase and re-enter the cell cycle between approximately 20 and 48 h after hatching (Ito and Hotta, 1992). During this secondary phase of proliferation, which lasts to the end of the larval stage, most neuroblasts generate an average of 150 postembryonic ("secondary") neurons (Bello et al., 2008). Similar to primary neurons, secondary neurons of a given lineage form coherent clusters of neuronal cell bodies and project axons which bundle together as the secondary axon tract (SAT). Secondary axon tracts form a stereotyped, conspicuous pattern that is visible from the larva through metamorphosis into the adult stage (Lovick et al., 2013; Wong et al., 2013). Differentiation of secondary

* Correspondence to: Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, 610 Charles E. Young Drive, 5009 Terasaki Life Sciences Bldg, Los Angeles, CA 90095-1606, USA.
E-mail address: volkerh@mcdb.ucla.edu (V. Hartenstein).

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neurons (i.e. sprouting of branches and formation of synapses) occurs during metamorphosis, along with remodeling of primary neurons; both secondary neurons and remodeled primary neurons form the adult brain circuitry.

The mechanism triggering the larval ("secondary") phase of proliferation involves signals derived from the surface glia surrounding the neuroblasts (Ebens et al., 1993). The insulin pathway, which links larval growth in general to the nutritional state, plays an important role in secondary neuroblast proliferation as well (Chell and Brand, 2010). Many aspects of how secondary neuroblast proliferation is initiated remain unknown. In particular, it is not clear whether and how the identity of a neuroblast influences the time point at which it enters mitosis. The time period over which neuroblasts start to divide lasts for more than 24 h, though the order in which neuroblasts resume proliferation and produce their respective secondary lineages has not been documented. In other words, in any given larva, some neuroblasts enter mitosis considerably earlier than others. Given the high degree of stereotypy of neuroblasts in the embryo (Urbach and Technau, 2003a; Younossi-Hartenstein et al., 1996), and of lineages and their SATs in the late larva (Pereanu and Hartenstein, 2006), we assumed that the birth order of secondary lineages is also highly invariant: a neuroblast of a given identity will always re-enter mitosis at the same time point. To test this hypothesis we used the drug hydroxyurea (HU), a compound known to arrest actively dividing cells, to ablate proliferating neuroblasts and therefore secondary neurons (lineages) they give rise to (de Belle and Heisenberg, 1994; Prokop and Technau, 1994). If our assumption is correct, applying HU at a specific time point should always affect the same set of lineages. We systematically administered short HU pulses during and after the 20–48 h period when neuroblasts enter their larval phase of proliferation and analyzed the effect on the development of secondary lineages in the late larval and adult brain using global markers for SATs (anti-Neurotactin/BP106, anti-Neuroglian/BP104), as well as several lineage-specific Gal4 lines.

Our data demonstrate that the time points at which secondary neuroblasts start to divide are indeed fairly stereotyped, allowing us to reconstruct a "birth calendar" for all lineages. Knowing the birth date of a lineage is of importance for future experiments targeting that particular lineage for ablation or lineage-specific manipulation by mosaic analysis. Aside from establishing lineage birth dates, our results also provide new insights into the degree of plasticity in *Drosophila* brain development. Trajectories of secondary axon tracts appear to be established largely independently of each other. Similarly, the structure of primary neurons in the larval and adult brain is mostly unaffected by the loss of secondary lineages. In contrast to the apparent rigid nature in which axonal trajectories are established, the final patterning of terminal arbors by secondary lineages appears to depend upon the presence of corresponding neuronal targets (loss of target tissue leads to the absence of terminal arbors by surviving secondary lineages in that region).

Materials and methods

Genetics

Flies were grown at 25 °C using standard fly media unless otherwise noted. *per*-Gal4 (Kaneko and Hall, 2000), *en*-Gal4 (Tabata et al., 1995), *ple*-Gal4 (TH-Gal4; Friggi-Grelin et al., 2003; #8848, Bloomington *Drosophila* Stock Center, University of Indiana, IN, USA), GH146-Gal4 (a gift from R.F. Stocker, University of Fribourg, Switzerland; Stocker et al., 1997), UAS-mcd8::GFP (Lee et al., 1999; #5137, BDSC).

Immunohistochemistry

Samples were fixed in 4% formaldehyde or 4% methanol-free formaldehyde in phosphate buffer saline (PBS, Fisher-Scientific, pH = 7.4; Cat No. #BP399-4). Tissues were permeabilized in PBT (PBS with 0.1–0.3% Triton X-100, pH = 7.4) and immunohistochemistry was performed using standard procedures (Ashburner, 1989). The following antibodies were provided by the Developmental Studies Hybridoma Bank (Iowa City, IA): mouse anti-Bruchpilot (Brp, 1:20), mouse anti-Neurotactin (BP106, 1:10), rat anti-DN-Cadherin (DN-EX #8, 1:20), and mouse anti-Neuroglian (BP104, 1:30). Secondary antibodies, IgG_1 (Jackson ImmunoResearch; Molecular Probes) were used at the following dilutions: Cy5-conjugated anti-rat Ig (1:100), Cy3-conjugated anti-mouse Ig (1:200), Cy5-conjugated anti-mouse Ig (1:250), Alexa 546-conjugated anti-mouse (1:500), DynaLight 649-conjugated anti-rat (1:400), Alexa 568-conjugated anti-mouse (1:500).

Hydroxyurea (HU) ablation experiments

Hydroxyurea (HU, Sigma) acts as a DNA-synthesis inhibitor which blocks the normal function of nucleotide reductase (Timson, 1975) and is lethal to S-phase cells (Furst and Mahowald, 1985). HU has been used in *Drosophila* to ablate adult muscle precursors (Broadie and Bate, 1991) as well as central brain neuroblasts (de Belle and Heisenberg, 1994; Stocker et al., 1997). Procedure for preparation of HU was adapted from Broadie and Bate (1991). HU was administered to fly larvae through the diet. Briefly, HU was dissolved in distilled water at a concentration of 50 mg/ml. The dissolved HU was then added to partially cool melted fly media to achieve a final concentration of 5 mg/ml. After thorough mixing, the HU media was poured onto 60 × 15 mm Petri dishes to cool. Food plates were made fresh (< 1 day beforehand) for each experiment.

To ablate neuroblasts, staged larvae were allowed to grow on standard media at 25 °C in Petri dishes until time of ablation. Larvae were quickly transferred via blunted forceps to food plates containing 5 mg/ml of HU for 4 h. This is sufficient time for the HU to accumulate to doses high enough to kill actively dividing neuroblasts (Broadie and Bate, 1991; Truman and Bate, 1988; White and Kankel, 1978). After 4 h, larvae were transferred to Petri dishes containing standard media and grown until dissected as either wandering L3 or adults. Fly stocks and larvae for experiments were grown at 25 °C.

Confocal microscopy

Staged *Drosophila* larval and adult brains labeled with suitable markers were viewed as whole-mounts by confocal microscopy [LSM 700 Imager M2 using Zen 2009 (Carl Zeiss Inc.); lenses: 40 × oil (numerical aperture 1.3)]. Complete series of optical sections were taken at 2-μm intervals. Captured images were processed by ImageJ or FIJI (National Institutes of Health, http://rsbweb.nih.gov/ij/ and http://fiji.sc/) and Adobe Photoshop.

Generation of three-dimensional models

Digitized images of confocal sections were imported into FIJI (Schindelin et al., 2012; http://fiji.sc/). Complete series of optical sections were taken at 2-μm intervals. Since sections were taken from focal planes of one and the same preparation, there was no need for alignment of different sections. Models were generated using the 3-dimensional viewer as part of the FIJI software package. Digitized images of confocal sections were imported using TrakEM2 plugin in FIJI software (Cardona et al., 2012). Surface renderings of larval brains stained with anti-Bruchpilot