Developmental Biology 356 (2011) 553–565

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Multipotent neural stem cells generate glial cells of the central complex through transit amplifying intermediate progenitors in *Drosophila* brain development

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ARTICLE INFO

Article history:
Received for publication 16 December 2010
Revised 27 May 2011
Accepted 11 June 2011
Available online 25 June 2011

Keywords:
Drosophila
Larval brain
Neuroblast lineages
Intermediate progenitor cells
Neuroglioblast
Gliogenesis

ABSTRACT

The neural stem cells that give rise to the neural lineages of the brain can generate their progeny directly or through transit amplifying intermediate neural progenitor cells (INPs). The INP-producing neural stem cells in *Drosophila* are called type II neuroblasts, and their neural progeny innervate the central complex, a prominent integrative brain center. Here we use genetic lineage tracing and clonal analysis to show that the INPs of these type II neuroblast lineages give rise to glial cells as well as neurons during postembryonic brain development. Our data indicate that two main types of INP lineages are generated, namely mixed neuronal/glial lineages and neuronal lineages. Genetic loss-of-function and gain-of-function experiments show that the *gcm* gene is necessary and sufficient for gliogenesis in these lineages. The INP-derived glial cells, like the INP-derived neuronal cells, make major contributions to the central complex. In postembryonic development, these INP-derived glial cells surround the entire developing central complex neuropile, and once the major compartments of the central complex are formed, they also delimit each of these compartments. During this process, the number of these glial cells in the central complex is increased markedly through local proliferation based on glial cell mitosis. Taken together, these findings uncover a novel and complex form of neurogliogenesis in *Drosophila* involving transit amplifying intermediate progenitors. Moreover, they indicate that type II neuroblasts are remarkably multipotent neural stem cells that can generate both the neuronal and the glial progeny that make major contributions to one and the same complex brain structure.

Introduction

Neural stem cells are the primary progenitor cells at different developmental stages that initiate the neural lineages comprising the differentiated neurons and glia of the brain (Kriegstein and Alvarez-Buylla, 2009). Analysis of stem cell-dependent neurogenesis in several model systems indicates that neural cells are not always produced directly from the primary progenitors, but can also be generated by secondary progenitors of more restricted potential (Götz and Huttner, 2005; Kriegstein et al., 2006). In mammals, neural stem cells can generate neural cells either directly through asymmetric division or indirectly through secondary progenitors which either divide only once to produce two neural progeny or divide more than once to amplify the number of neural progeny further (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004, 2007).

Remarkably similar findings have been obtained in studies of neurogenesis in the developing CNS of *Drosophila*. There, neural stem cell-like primary progenitors called neuroblasts can also generate neurons directly through asymmetric division or, more typically, indirectly through ganglion mother cells (GMC) which divide only once to generate two postmitotic progeny (Doe, 2008; Knoblich, 2008; Skeath and Thor, 2003; Technau et al., 2006). Moreover, recently a third type of neurogenesis has been discovered in the *Drosophila* central brain where identified neuroblasts generate intermediate neural progenitor cells (INPs) which undergo several rounds of asymmetric divisions, each of which results in INP self-renewal and generation of a GMC that produces two neural progeny (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). These INPs have also been referred to as IPs, transit amplifying GMCs, or secondary neuroblasts (Weng and Lee, 2010.) Through the resulting amplification of proliferation these INP producing "type II" neuroblasts generate large neural lineages that contribute to major neuropile substructures of the brain such as the central complex (Bayraktar et al., 2010; Izergina et al., 2009; Pereanu et al., 2011).

In mammalian brain development, neural stem cells can give rise to both neurons and glial cells, however the neurogenic phase and the gliogenic phase of these primary progenitors are generally separate (Miller and Gauthier, 2007; Miyata et al., 2010). Neural stem cells that can generate both neurons and glia are also found in *Drosophila* (Van De Bor and Giangrande, 2002). These so-called neuroglioblasts have been identified on a single-cell basis in the embryonic ventral nerve cord, and together with glioblasts, which only generate glial cells, they give rise to several subtypes of glial cells in the ventral CNS (Beckervordersandforth

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doi:10.1016/j.ydbio.2011.06.013

et al., 2008; Bossing et al., 1996; Broadus et al., 1995; Schmidt et al., 1997). In the ventral nerve cord, gliogenesis is controlled by the *glial cells missing (gcm)* gene that acts as a key switch-like regulator in the development of all embryonic glial cells except those generated from mesectodermal precursors (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996).

Much less is known about the developmental origins of the glial cells in the *Drosophila* larval brain. The glial cells of the early larval brain are thought to be generated by a few neuroglioblasts during embryogenesis (Hartenstein et al., 1998). However, most of the glial cells in the adult central brain are generated postembryonically, and during larval development a marked increase in glial cell number occurs (Pereanu et al., 2005). While some of these glial cells apparently arise by mitosis of other glial cells, the bulk of the postembryonically added glial cells is thought to be generated through the proliferation of neuroglioblasts, which, however, have not yet been identified (Awasaki et al., 2008; Pereanu et al., 2005). This lack of identification of the postulated postembryonic neuroglioblasts has been a major obstacle for understanding the mechanisms by which glial cells are generated during postembryonic development of the central brain.

Here we show that identified type II neuroblasts have neuroglioblast function during postembryonic development of the central brain. We use genetic lineage tracing and clonal analysis to demonstrate that the INPs of type II neuroblast lineages can give rise to glial cells as well as neurons. Our data indicate that two main types of INP-derived lineages are generated postembryonically, namely mixed neuronal/glial lineages and neuronal lineages. Moreover, they show that INP-derived glial cells, like INP-derived neuronal cells from the same type II neuroblast lineages, make major contributions to the developing neuropile of the central complex. They surround the entire developing central complex neuropile, and once its major compartments are formed, they also delimit each of these. During this process, the population of INP-derived glial cells associated with central complex undergoes clonal expansion through local proliferation. Taken together, these findings uncover novel mechanisms for neurogliogenesis in *Drosophila* involving the transit amplifying intermediate progenitors of type II lineages. Thus, type II neuroblasts are remarkably multipotent neural stem cells that can generate both the neuronal and the glial progeny of one and the same complex brain structure.

Materials and methods

Fly stocks, MARCM, and G-Trace analysis

Flies were maintained on standard cornmeal medium at 25 °C unless noted otherwise. To visualize the potentially glial offspring of neuroblasts, we used *insc-Gal4^{MZ1407}*, *UAS-mCD8GFP^{LL5}* homozygous flies, or mated them to *gcm-lacZ^{rA87}/CyO, act-gfp^{JMR1}*. For *erm-Gal4* (R09D11; Pfeiffer et al., 2008) G-Trace (flp-out and real-time; Evans et al., 2009) expression patterns we mated *UAS-flp, ubi>stop>nGFP, UAS-RFP/CyO, act-gfp^{JMR1}*; *erm-Gal4* to *gcm-lacZ^{rA87}/CyO, act-gfp^{JMR1}; erm-Gal4*. For membrane labeling of *erm-Gal4* progeny, we mated *UAS-flp*; *UAS-mCD8GFP^{LL5}*; *erm-GAL4* to *UAS-mCD8GFP^{LL5}*; act>*,*CD2>Gal4* (Pignoni and Zipursky, 1997). To generate wild type MARCM (Lee and Luo, 1999) clones, we mated *y w hs-flp^{1}; tubP-Gal4, UAS-mCD8GFP^{LL5}/CyO, act-gfp^{JMR1}; FRT82B, tub-Gal80^{LL3}* (Bello et al., 2003) to *gcm-lacZ^{rA87}/CyO, act-gfp^{JMR1}; FRT82B* males. To generate clones misexpressing *gcm*, we mated *elav-Gal4^{C155}, hs-flp^{1}; UAS-mCD8GFP^{LL5}/CyO, act-gfp^{JMR1}; FRT82B tub-Gal80^{LL3}* to *UAS-gcm^{m24a}/CyO, act-gfp^{JMR1}; FRT82B* (*UAS-gcm^{m24a}* from Bernardoni et al., 1998). To induce *gcm* loss-of-function clones, we mated *y w hs-flp^{1}; FRT40A, tubP-Gal80^{LL10}/CyO, act-gfp^{JMR1}; tubP-Gal4^{LL7}, UAS-mCD8GFP^{LL6}* (Bello et al., 2006) to *FRT40A, gcm^{N7-4}/CyO, act-gfp^{JMR1}*. Eggs were collected for 2–4 h, grown to first larval instar (22–30 h after egg laying, AEL), plates immersed in a 37 °C waterbath for 5 min (sparse wild type clones) or up to 30 min (other clones), and grown to the desired stage at 25 °C. When recovering clones from wandering L3 larvae, some bottles were raised at 18 °C during third instar to delay development until dissection. When recovering clones from earlier stages, larvae were grown at 25 °C throughout, and kept at a maximum density of 170 larvae per bottle to avoid developmental delay due to food competition and to ensure exact staging.

Immunohistochemistry and in situ hybridization

Brains were dissected in ice-cold PBS and fixed in 2% Paraformaldehyde for 30–60 min at room temperature, washed several times in PBS/0.5% Triton X-100, and preincubated in PBS containing 0.5% Triton X-100 and 10% normal goat serum. Antibodies were incubated overnight at 4 °C. We used chicken anti-GFP 1:500 (ab13970, Abcam, Cambridge, UK), rabbit anti-beta-Galactosidase 1:500 (55976, MP Biomedicals, Solon, Ohio, USA), mouse anti-Neurotactin 1:20 (BP106, DSHB, Iowa City, Iowa, USA), rat anti-Elav 1:30 (7E8A10, DSHB), mouse anti-Repo 1:30 (8D12, DSHB), rabbit anti-Repo 1:400 (kindly provided by Veronica Rodrigues), rabbit anti-phospho histone H3 (Ser10, 06-570, Millipore, Temecula, CA, USA), rat anti-Deadpan and rabbit anti-Asense (both kind gifts from Cheng-Yu Lee), and Alexa-conjugated secondary antibodies 1:300 (A11039, A21247, A11036, A11031, A21244, Molecular Probes, Eugene, OR, USA). In situ hybridization was performed as described previously (Soustelle et al., 2007).

Identification of dorsomedial type II lineages

DM1 was uniquely identified in Nrt/BP106 staining as the dorsoanterior-most large lineage in late third instar brains. DM5 was identified by a unique *gcm-lacZ* and Repo-positive cell cluster within the lineage (Figs. 1D, 5E). Other DM lineages were identified by their position relative to DM1 and DM5, their very large cell number (Bello et al., 2008), complex axon branching pattern (Izergina et al., 2009), and location at the posterior/medial brain surface. In younger larvae, DM1–3 lineages were recognized by their similar shapes and positions compared to those in wandering larvae. In addition, they were confirmed to be Type II lineages by weak or absent Asense staining in the neuroblast, the presence of numerous Asense- or Deadpan-positive cells apart from the neuroblast, or equally numerous Prospero- and Elav-negative cells throughout the lineage.

Microscopy and image processing

Fluorescent images were recorded on a Leica TCS SP5 confocal microscope, and processed using Fiji (Schindelin, 2008) or ImageJ (Rasband, 1997–2008). All adjustments were linear and were performed on whole images. Cells were counted using the CellCounter plugin for Fiji/ImageJ (Kurt De Vos).

Results

Postembryonic development of neurons and glial cells in the central brain

During postembryonic development, the majority of the neurons (95%) that make up the adult central brain are generated by approximately 100 pairs of neural stem cell-like neuroblasts, each of which generates a specific lineage of neural progeny (reviewed in Hartenstein et al., 2008). Fig. 1A shows the neuroblast-derived lineages in the central brain of the third larval instar. In addition to neurons, a large number of glial cells are also generated postembryonically in the central brain (Fig. 1B, C). Newly generated glia express both *gcm-lacZ^{rA87}* and the glial identity marker Repo, whereas older (or *gcm*-independent) glia express only Repo (Fig. 1B, C).

Among the 100 central brain neuroblasts in each brain hemisphere, there are a total of eight identified type II neuroblasts, and six of these occupy most of the posterior medial edge of the hemisphere (Fig. 1D, E). Because they are easier to identify, most studies of type II