Developmental Biology 393 (2014) 255–269

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Developmental regulation of glial cell phagocytic function during *Drosophila* embryogenesis

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ARTICLE INFO

Article history:
Received 7 May 2014
Received in revised form
7 July 2014
Accepted 10 July 2014
Available online 18 July 2014

Keywords:
Drosophila
Glia
Phagocytosis
Apoptosis
SIMU
Development

ABSTRACT

The proper removal of superfluous neurons through apoptosis and subsequent phagocytosis is essential for normal development of the central nervous system (CNS). During *Drosophila* embryogenesis, a large number of apoptotic neurons are efficiently engulfed and degraded by phagocytic glia. Here we demonstrate that glial proficiency to phagocytose relies on expression of phagocytic receptors for apoptotic cells, SIMU and DRPR. Moreover, we reveal that the phagocytic ability of embryonic glia is established as part of a developmental program responsible for glial cell fate determination and is not triggered by apoptosis per se. Explicitly, we provide evidence for a critical role of the major regulators of glial identity, *gcm* and *repo*, in controlling glial phagocytic function through regulation of SIMU and DRPR specific expression. Taken together, our study uncovers molecular mechanisms essential for establishment of embryonic glia as primary phagocytes during CNS development.

Introduction

Efficient recognition and elimination of apoptotic cells through phagocytosis is crucial for normal development of multicellular organisms. Apoptotic cell clearance is accomplished by two types of phagocytes: 'professional' macrophages and immature dendritic cells and 'non-professional' tissue-resident neighboring cells, whose role is critical during development (Elliott and Ravichandran, 2008; Henson and Hume, 2006; Scheib et al., 2012). Phagocytes must recognize apoptotic particles with high level of specificity in order to specifically remove apoptotic cells but not living normal cells. This very precise recognition is achieved through transmembrane phagocytic receptors or secreted bridging molecules, which recognize 'eat me' signals on apoptotic surfaces (Kinchen and Ravichandran, 2007, 2008a, 2008b; Lauber et al., 2004; Ravichandran, 2011; Ravichandran and Lorenz, 2007; Stuart and Ezekowitz, 2005).

In mammals, a large number of transmembrane receptors and soluble bridging molecules have been shown to play a role in recognition and engulfment of apoptotic particles (Hanayama et al., 2002; Miyanishi et al., 2007; Park et al., 2007, 2008, 2009). Importantly, most of these are exclusively expressed in phagocytic cell populations. However, the molecular mechanisms controlling their specific expression remain elusive. Several phagocytic receptors for apoptotic cells are also known in *Drosophila*. These receptors show highly specific expression in phagocytic cell populations during embryogenesis. For example, the CD36 homolog Croquemort (CRQ) is expressed mostly in professional phagocytes, the macrophages (Franc et al., 1999). Two other receptors, Draper (DRPR) and Six Microns Under (SIMU), are expressed both in macrophages and in glia, the non-professional phagocytes of the central nervous system (CNS) (Freeman et al., 2003; Kurant et al., 2008).

During late embryogenesis a large number of neurons die through apoptosis and embryonic glia function as the main phagocytes in the CNS, which efficiently remove them (Freeman et al., 2003; Kurant et al., 2008). Although different functions of glia have recently received great attention (Allen and Barres, 2005; Barker and Ullian, 2010; Barres, 2008; Chotard and Salecker, 2004; Edenfeld et al., 2005; Farina et al., 2007; Freeman, 2006; Freeman and Doherty, 2006; Halassa and Haydon, 2010; Kurant, 2011; Logan and Freeman, 2007; Pfrieger, 2010; Vilhardt, 2005), mechanisms responsible for preparing glia to be potent phagocytic cells remain poorly understood.

We have previously shown that SIMU, which is required for recognition and engulfment of apoptotic neurons by glial cells, is expressed exclusively during stages of developmental apoptosis in *Drosophila*: mid-to-late embryogenesis, pupae and early adult (Kurant et al., 2008). How this specific expression, which entirely correlates with developmentally programmed cell death, is regulated

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http://dx.doi.org/10.1016/j.ydbio.2014.07.005
0012-1606/

remains unclear. In the work presented here, we demonstrate that the apoptotic process itself does not affect expression levels of phagocytic receptors SIMU and DRPR in glia but this specific expression is part of a developmental program responsible for glial cell determination. Specifically, we show that a master regulator of glial cell fate, *glial cells missing* (*gcm*), which is expressed in precursors of macrophages and early developing glia (Bernardoni et al., 1997; Hosoya et al., 1995; Jones et al., 1995), induces the expression of SIMU and DRPR in glia, but not in macrophages. We also demonstrate that a key regulator of lateral glia development, *reversed polarity* (*repo*) (Halter et al., 1995; Xiong et al., 1994), is required for DRPR but not SIMU expression in glia. Moreover, we provide evidence that GCM directly regulates *simu* expression in glia through its specific binding sites, although *drpr* glial expression requires *repo*. Altogether, our study uncovers molecular mechanisms responsible for establishment of glial phagocytic ability during development.

Results

Developmental apoptosis during embryogenesis does not affect expression of the phagocytic receptors SIMU and DRPR

The strong correlation between SIMU expression and developmental apoptosis (Kurant et al., 2008) prompted us to test whether apoptosis itself promotes expression of phagocytic receptors during embryonic development. In order to address this question, we first examined the level of SIMU and DRPR receptors in embryos lacking developmental apoptosis. A specific deletion of a genomic region containing three pro-apoptotic genes *reaper*, *grim* and *hid* (*H99*) completely abrogates caspase activation in the embryo, resulting in lack of developmental apoptosis (White et al., 1994). We monitored caspase activation in the embryonic CNS by staining with an anti-cleaved caspase 3 antibody (CM1), which specifically labels apoptotic particles in wild type embryo (Fig. 1A) and shows no reactivity in *H99* embryos (Fig. 1B). In *H99* mutant embryos we tested protein expression using anti-SIMU (Fig. 1C, D, G, and H) and anti-DRPR (Fig. 1E–H) antibodies and detected SIMU and DRPR proteins on glial membranes similarly to wild type staining which has been described previously (Kurant et al., 2008, Fig. 1G and H), indicating that lack of apoptosis does not affect protein levels of phagocytic receptors. We also did not notice any change in GFP expression of the *simu-cytGFP* reporter, which contains a 2 kb region upstream of *simu* translation start site fused to cytoplasmic GFP, which completely recapitulates SIMU embryonic expression (Kurant et al., 2008, Fig. 1I and J).

Moreover, we measured the levels of *simu* and *drpr* transcripts by qRT-PCR analysis. We detected similar levels of *simu* cDNA in the mutant embryos compared to wild type embryos (Fig. 1K), suggesting that lack of developmental apoptosis does not affect *simu* transcriptional levels. Similarly, comparable levels of *drpr* cDNA were detected in *H99* and wild type embryos (Fig. 1K), demonstrating that *drpr* transcript levels are not affected by lack of apoptosis as well.

To explore, reciprocally, whether elevated apoptosis stimulates expression of phagocytic receptors during embryogenesis, we examined *simu* and *drpr* expression in embryos with high levels of apoptosis. To induce excess apoptosis we used *elavGal4*::*hid* transgenic embryos, which show upregulated neuronal apoptosis. Apoptotic rates were assessed by staining with the CM1 antibody (Fig. 2A and B). When we tested protein expression of SIMU (Fig. 2A′, A″, B′, and B″) and DRPR (Fig. 2E and F) in the *elavGal4*::*hid* embryos we observed an abnormal shape of glial cells labeled with anti-SIMU (Fig. 2B″) and anti-DRPR (Fig. 2F) antibodies. This glial shape is a result of increased glial engulfment of higher amounts of apoptotic particles in the *elavGal4*::*hid* embryos (Fig. 2B) as compared to wild type (Fig. 2A). However, similar levels of SIMU and DRPR proteins were detected (Fig. 2A″, B″, E, and F), suggesting that increased apoptosis does not affect protein levels of phagocytic receptors. Moreover, since *elavGal4* is transiently expressed in embryonic glia (Berger et al., 2007), in order to test whether the transient expression of HID in glia affected glial cell number, we quantified REPO-labeled glial cells in entire *elavGal4*::*hid* embryonic CNS (Fig. 2D) and compared it to wild type controls (Fig. 2C). No significant difference in glial cell number was detected between *elavGal4*::*hid* and control embryos (Fig. 2G), demonstrating no glial death in these embryos.

In addition, qRT-PCR analysis of *elavGal4*::*hid* and control embryos showed that increased apoptosis did not affect the transcriptional levels of *simu* and *drpr* during late embryogenesis (Fig. 2H). Since the expression levels of *simu* and *drpr* are relatively high in normal embryos, it may be difficult to detect an increase in their expression at this developmental stage. We, therefore, induced ectopic apoptosis during larval stages when normally no apoptosis takes place (Fig. 2I). In wild type larvae *simu* expression levels are undetectable at this stage (Kurant et al., 2008, Fig. 2K), whereas *drpr* expression is lower, as compared to embryogenesis (Flybase expression data, Figs. S1 and 2I′).

To bypass HID-induced neuronal apoptosis during embryogenesis, which is lethal, we conditionally expressed *hid* in larval neurons (*elavGal4*::*hid*) using the temperature-sensitive Gal80 repressor (*tubGal80^{ts}*). At 18 °C *tubGal80^{ts}* is expressed in all embryonic tissues and prevents *elavGal4* from induction of *hid* neuronal expression. At 29 °C Gal80 is inactivated and *elavGal4* is derepressed leading to expression of *hid* specifically in larval neurons. We placed the progeny *elavGal4*::*hid*; *tubGal80* at 18 °C until the 2nd instar larvae stage and then shifted them to 29 °C for 24 h. Dissected larval brains of the *tubGal80^{ts}*; *elavGal4*::*hid* third instar larvae were stained with the CM1 antibody to confirm induction of apoptosis (Fig. 2J and J″), and with specific antibodies for SIMU and DRPR in order to evaluate their protein expression levels. No detectable expression of SIMU was found in these brains (Fig. 2L) and no difference in DRPR expression was obtained as compared to wild type (Fig. 2I′, I″, J′, and J″), suggesting that elevated apoptosis did not affect SIMU and DRPR expression in larval brains. Moreover, the progeny *elavGal4*::*hid*; *tubGal80* and control *elavGal4*; *tubGal80* were subjected to qRT-PCR analysis following incubation at 29 °C as described above. We found no increase in *drpr* expression compared to wild type (Fig. 2H) and no detectable *simu* expression in the *elavGal4*::*hid; tubGal80* larvae. These results indicate that induced apoptosis does not influence *simu* and *drpr* expression in larval stages, similarly as during embryogenesis. Altogether, these data suggest that expression of phagocytic receptors (in glia and macrophages) is not affected by levels of apoptosis, raising the possibility that their expression is part of the developmental program responsible for phagocytic cell fate determination.

glial cells missing (gcm) differentially affects expression of SIMU and DRPR in haemocytes and glia

gcm is expressed in precursors of 'professional' macrophages, haemocytes, and early lateral glia (Bernardoni et al., 1997; Hosoya et al., 1995; Jones et al., 1995), making it a promising candidate for regulation of phagocytic ability of two phagocytic cell populations, macrophages and glia. In addition, two GCM putative binding sites have been identified (Genomatix software) within a 2 kb region upstream of *simu* translation start site, which completely recapitulates SIMU embryonic expression (Kurant et al., 2008, Fig. 3A).