Developmental Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Developmental Biology

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

Original research article

Bergmann glial Sonic hedgehog signaling activity is required for proper cerebellar cortical expansion and architecture

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ARTICLE INFO

Keywords:
Bergmann glia
Sonic hedgehog
Cerebellum
Neuronal-glial relationships

ABSTRACT

Neuronal-glial relationships play a critical role in the maintenance of central nervous system architecture and neuronal specification. A deeper understanding of these relationships can elucidate cellular cross-talk capable of sustaining proper development of neural tissues. In the cerebellum, cerebellar granule neuron precursors (CGNPs) proliferate in response to Purkinje neuron-derived Sonic hedgehog (Shh) before ultimately exiting the cell cycle and migrating radially along Bergmann glial fibers. However, the function of Bergmann glia in CGNP proliferation remains not well defined. Interestingly, the Hh pathway is also activated in Bergmann glia, but the role of Shh signaling in these cells is unknown. In this study, we show that specific ablation of Shh signaling using the tamoxifen-inducible $TNC^{YFP\text{-}CreER}$ line to eliminate Shh pathway activator Smoothened in Bergmann glia is sufficient to cause severe cerebellar hypoplasia and a significant reduction in CGNP proliferation. $TNC^{YFP\text{-}CreER}$; $Smo^{F/-}$ (Smo^{CKO}) mice demonstrate an obvious reduction in cerebellar size within two days of ablation of Shh signaling. Mutant cerebella have severely reduced proliferation and increased differentiation of CGNPs due to a significant decrease in Shh activity and concomitant activation of Wnt signaling in Smo^{CKO} CGNPs, suggesting that this pathway is involved in cross-talk with the Shh pathway in regulating CGNP proliferation. In addition, Purkinje cells are ectopically located, their dendrites stunted, and the Bergmann glial network disorganized. Collectively, these data demonstrate a previously unappreciated role for Bergmann glial Shh signaling activity in the proliferation of CGNPs and proper maintenance of cerebellar architecture.

1. Introduction

Cerebellar development proceeds in a tightly regulated manner, requiring the proper balance of neural progenitor cell expansion and differentiation to form a characteristically organized structure. However, our understanding of the cellular relationships and signaling pathways that contribute to this balance is incomplete. A cell type integral to development of the cerebellum is the cerebellar granular neuron precursor (CGNP), which occupies a transient layer on the outer surface of the cerebellum from embryonic day 14 to two weeks postnatally in mice. CGNPs proliferate in response to Sonic hedgehog (Shh) ligand, which is secreted by neighboring Purkinje cells (PC). The Shh signal is transduced in CGNPs by the Smoothened (Smo) transmembrane protein to initiate production of activator forms of the Gli transcription factors (Goodrich et al., 1996; Ingham and McMahon, 2001; Marigo et al., 1996; Varjosalo and Taipale, 2008; Fuse et al., 1999). After exiting mitosis, differentiated granular neurons migrate inward, past the PC layer, where they populate the internal granular layer (IGL). CGNPs are the presumed cell-of-origin for the Shh-driven subset of the malignant pediatric brain tumor medulloblastoma; thus understanding the cellular and molecular factors that govern their proliferation is critical.

Neuronal progenitors including CGNPs are often in close contact with glial cells (Shiga et al., 1983; Buffo and Rossi, 2013), however relatively little attention has been given to the function of neuron-glial interactions in the cerebellum. Specialized, unipolar astrocytes called Bergmann glia (BG) are present in the cerebellum and originate from radial glia of the cerebellar ventricular zone. As early postnatal cerebellar development proceeds, BGs migrate behind PCs, ultimately aligning their cell bodies in the same single-celled layer. Their characterized functions in the cerebellum are three-fold. First, BG radial fibers extend to the pial surface shortly after birth where their endfeet contact the basement membrane (Rakic, 1971; Yamada et al., 2000). The endfeet adhere to one another to form a glia limitans over the cerebellum (Das, 1976), providing structural support as the cerebellar plate expands (Hausmann and Sievers, 1985; Sievers and Pehlemann, 1986; Sievers et al., 1981). Second, as granular neurons differentiate and begin to migrate inwards to form the internal granular

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https://doi.org/10.1016/j.ydbio.2018.05.015
Received 20 February 2018; Received in revised form 7 May 2018; Accepted 18 May 2018

layer, BG radial fibers function as guides to the closely aligned CGNP cell bodies (Rakic, 1971). And last, BG radial fibers synapse on PC dendrites; it has been proposed that BGs contribute to PC dendritic elaboration (Lippman et al., 2008; Yamada et al., 2000) and stabilization of neuronal synaptic connections (Iino, 2001; Yue, 2005).

BGs belong to a select group of specialized astroglia that retain radial glial-like morphology postnatally and into adulthood; members of this group include BGs, Muller cells in the retina, and tanycytes of the hypothalamus (Rakic, 2003). Importantly, while both Muller cells and tanycytes have been demonstrated to have neurogenic potential and contribute to neurogenesis in their respective regions (Haan et al., 2013; Surzenko et al., 2013; Robins et al., 2013), BGs have not been found to display such features. Rather, mice with BG defects during development exhibit altered cerebellar layering, neuronal migration, synaptic connectivity, and a disrupted pial membrane (Belvindrah et al., 2006; Graus-Porta et al., 2001; Wang et al., 2011; Eiraku et al., 2005; Komine et al., 2007; Weller et al., 2006). The contribution of BGs to neuronal specification and proliferation in the cerebellum has not been extensively studied.

Many signaling pathways are important for the formation and maintenance of BGs. Genetic studies using mice that lack Notch pathway components have demonstrated the pathway to be integral for BG specification, maturation, and monolayer formation (Eiraku et al., 2005; Komine et al., 2007; Weller et al., 2006). Other studies have shown that PTEN and integrin-linked kinase play roles in BG differentiation (Yue, 2005; Belvindrah et al., 2006), whereas APC maintains BG morphology (Wang et al., 2011) and the guanine nucleotide exchange factor Ric-8a regulates BG basement membrane adhesion (Ma et al., 2012). Transcriptional profiling studies of BGs also identified the Wnt and TGFß signaling pathways as developmentally upregulated in BG though their function in BG has yet to be identified (Koirala and Corfas, 2010). Interestingly, BGs have been shown to be capable of responding to Purkinje-derived Shh signals in postnatal stages through adulthood (Corrales et al., 2004, 2006). It has been observed that Shh signaling induces the glial differentiation of immature postnatal mouse astroglia in vitro (Dahmane and Ruiz i Altaba, 1999). In addition, inhibition of Shh activity using 5E1 hybridoma cells injected into chick embryos at early stages resulted in massive perturbations of cerebellar development, including a concomitant reduction in BLBP+ BG (Dahmane and Ruiz i Altaba, 1999) (Dahmane and Ruiz i Altaba, 1999). However, the role of Shh signaling activity in BG in vivo and its consequences for cerebellar development are not well understood. Understanding how BG contribute to CGNP proliferation and thus overall architecture of the cerebellum can shed light on basic developmental processes and have implications for cerebellar diseases that derive from aberrant Shh signaling and neuronal-glial relationships.

In this study, we spatially and temporally alter Shh signaling activity in postnatal BG. Mice in which Shh activator Smoothened (Smo) is postnatally ablated in BG demonstrate an obvious reduction in cerebellar size within two days of ablation of Shh signaling. Surprisingly, mutant CGNPs exhibit severely reduced proliferation and increased differentiation accompanied by a loss of Shh activity, suggesting a novel role for the BG-CGNP interaction in promoting CGNP precursor proliferation. Interestingly, Wnt signaling is ectopically elevated in mutant CGNPs concomitant with a reduction in EGL area, suggesting that this pathway is involved in cross-talk with the Shh pathway in regulating CGNP proliferation. In addition, loss of Shh signaling in BG leads to disrupted PC laminar organization and dendritic arborization as well as BG fiber morphology, indicating that BG-Shh signaling activity contributes to the maintenance of proper cerebellar laminar formation. Collectively, these data show a previously unappreciated role for BG Shh signaling activity in the proliferation of CGNPs and preservation of cerebellar architecture, thus leading to a new level of understanding of the neuronal-glial relationship in the cerebellum.

2. Materials and methods

2.1. Animals and tamoxifen administration

Mice of the following genetic lines, of either sex, were used in the study: *Gli1*^{nlacZ} (Bai et al., 2002), *TNC*$^{YFP\text{-}CreER}$ (Fleming et al., 2013), *Smo*$^{F/F}$ (Long et al., 2001), *Bat-gal* (Maretto et al., 2003), *Math1*^{CreER} (Machold and Fishell, 2005), *R26R*^{eYFP} (Srinivas et al., 2001), *tdTomato* (Madisen et al., 2009), *L7-Cre* (Lewis et al., 2004) and *Shh*$^{F/F}$ (Lewis et al., 2001). Tamoxifen (Sigma) was dissolved to a final concentration of 2 mg/ML in corn oil (Sigma). Postnatal *TNC*$^{YFP\text{-}CreER}$; *tdTomato*, *TNC*$^{YFP\text{-}CreER}$; *R26R*^{eYFP}, *TNC*$^{YFP\text{-}CreER}$; *Smo*$^{F/-}$ (*Smo*^{cko}), *Math1*^{CreER}; *Smo*$^{F/-}$, and WT littermates received 50 µL of tamoxifen by intraperitoneal injection on P1 and P2 or on P4 and P5 where noted.

2.2. Tissue processing and Immunohistochemistry

For animals younger than P30, brains were dissected and fixed in 4% paraformaldehyde for either 4–6 h or O/N at 4 °C. Animals P30 and older received 50 µL intraperitoneal injections of Ketamine and received ice-cold PBS via transcardial perfusion followed by 4% paraformaldehyde. Brains were collected and submersion fixed in 4% paraformaldehyde O/N at 4 °C. These tissues were either processed for frozen embedding in OCT compound or processed for paraffin embedding. Frozen tissues were sectioned on a Leica cryostat at 10 µm, paraffin embedded tissues were cut at 5 µm. Immunohistochemistry were performed as previously described (Huang et al., 2009, 2010). The following primary antibodies were used on frozen and/or paraffin tissue sections: chicken α-β-Gal (ICL), rabbit α-β-Gal (ICL), rabbit α-BLBP (Abcam), rabbit α-GFAP (Abcam), chicken α-GFP (Aves), guinea pig α-Gli2 (Qin et al., 2011), rabbit α-Calbindin (Swant), rabbit α-phospho-Histone-3 (Upstate Cell Signaling), mouse α-NeuN (Millipore), mouse α-Parvalbumin (Sigma), rabbit α–3-PGDH (Thermo-Scientific), rabbit α-Sox2 (Millipore), mouse α-Laminin (Thermo-Scientific), rabbit α-p27^{Kip1} (BD Transduction Labs). For bright-field staining, species-specific HRP-conjugated secondary antibodies (Invitrogen) were used followed by incubation in DAB reaction (Invitrogen) or alkaline-phosphatase (Invitrogen). Double-labeling fluorescence immunohistochemistry was performed using species-specific, AlexaFluor-tagged secondary antibodies Alexa 488, Alexa 568, and Alexa 647 (Invitrogen) followed by counterstaining with To-pro3 iodide (Invitrogen).

2.3. X-Gal and in situ hybridization

X-Gal staining for β-Galactosidase activity was performed on post-fixed, frozen sections according to standard protocols. Section in situ hybridizations were performed using digoxygenin-labeled riboprobes as previously described (Li et al., 2006, 2008). Riboprobes were synthesized using the digoxygenin RNA labeling kit (Roche). The following cDNAs were used as templates for synthesizing digoxygenin-labeled riboprobes: *Shh* and *Sfrp1 (*gift of Paula Bovolenta, Centro de Biologia Molecular Universidad Autonoma Madrid, Madrid, Spain).

2.4. CGNP and cerebellar isolation and western blotting

For CGNP isolation, P4 or P5 cerebella from CD1 or Smo ^{CKO} mice were dissected into calcium-free Hanks buffered saline solution (Mediatech) supplemented with 6 g/L D-glucose. The meninges were stripped and pooled cerebella dissociated with Accutase (Gibco) and trituration. Cells were pelleted and resuspended in Neurobasal A-medium containing 250 µM KCl, 500 µL 100 × GlutaMAX I, 500 µL 100 × penicillin-streptomycin, and 10% FBS. Cells were passed through a 70 µm filter and incubated for two times 20 min on poly-D-lysine coated plates. Following the settling step, the cells remaining in the media were considered the CGNP fraction and were collected, pelleted,