

The Purkinje neuron: A central orchestrator of cerebellar neurogenesis

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Within the cyto-architecture of the brain is an often complex, but balanced, neuronal circuitry, the successful construction of which relies on the coordinated generation of functionally opposed neurons. Indeed, deregulated production of excitatory/inhibitory interneurons can greatly disrupt the integrity of excitatory/inhibitory neuronal transmission, which is a hallmark of neurodevelopmental disorders such as autism. Recent work has demonstrated that the Purkinje neuron, the central integrator of signaling within the cerebellar system, acts during development to ensure that neurogenesis occurring in spatially opposed domains reaches completion by transmitting the Sonic hedgehog ligand bi-directionally. In addition to a classic role in driving granule cell precursor proliferation, we now know that Purkinje neuron-derived Sonic hedgehog is simultaneously disseminated to the neonatal white matter. Within this neurogenic niche a lineage of Shh-responding stem and progenitor cells expand pools of GABAergic interneuron and astrocyte precursors. These recent findings advance our understanding of how Purkinje neurons function dynamically to oversee completion of a balanced cerebellar circuit.

Keywords: axon, cerebellum, GABAergic interneuron, neuronal stem cell, Purkinje neuron, sonic hedgehog, white matter

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have only recently become somewhat clear. The Sonic hedgehog pathway has been implicated as a key mediator in several phases of cerebellar ontogeny, but was initially identified as the principal driver of cortical growth. Cerebellar expansion is powered by the rapid clonal divisions of granule cell precursors (GCPs), which Purkinje neuron (PN)-derived Shh induces (Dahmane and Ruiz i Altaba, 1999; Lewis et al., 2004; Wallace, 1999; Wechsler-Reya and Scott, 1999).

PNs are situated many cell diameters away from recipient GCPs, and are thought to transmit Shh ligand outwardly along their dendritic axis to the superficial external granule cell layer where GCPs transiently reside (Lewis et al., 2004). In this manner, by governing the production of granule cells, PNs ensure that the major excitatory component of the cerebellar system is established (Sillitoe and Joyner, 2007). However, this outward signaling axis only accounts for the generation of one major element that feeds back on PNs, which must be counterbalanced by an appropriate opposing force. Functional opposition is provided, in part, by GABAergic inhibitory interneurons occupying the molecular layer (ML), basket and stellate cells, whose activity PNs also integrate to synchronize the cerebellar circuit.

The earliest born GABAergic cell types emerge during embryogenesis from radial glial stem cell-like cells residing in the ventricular zone (VZ) neuroepithelium (Altman, 1997), the establishment of which utilizes an extracerebellar Shh signal (Huang et al., 2010). However, the bulk of inhibitory interneurons are generated in a neurogenic compartment occupying the neonatal presumptive white matter (PWM) (Maricich et al., 1999; Weisheit et al., 2006; Zhang and Goldman, 1996).

Since the pioneering days of Ramon y Cajal, the cerebellum has provided a conduit for fundamental advancements in neurobiology. Many of the first morphological descriptions of neurons and glia detailed their specific connectivity and cortical placement within the cerebellum. However, our understanding of the neurogenic zones responsible for producing such exquisitely balanced cellular diversity, particularly the key niche signals,

The key signal(s) acting to regulate GABAergic neurogenesis within the PWM niche were unknown until our laboratory identified a lineage of Shh-responding progenitor cells within this domain (Fleming et al., 2013).

Prior work identified a neuronal stem cell (NSC) population occupying the neonatal PWM that are surface antigen CD133 (Prominin-1)-positive, lineage marker-negative (Lee et al., 2005). These NSCs were capable of generating both neurons and glia following in vitro differentiation and transplantation into the neonatal cerebellar cortex, and may be lineage-related to multipotent hGFAP⁺ progenitor cells also known to reside in the PWM (Silbereis et al., 2009). We were able to expand upon these findings by showing that PWM CD133⁺ NSCs also express low-level YFP driven by Tenascin-C (Tnc), an extracellular matrix glycoprotein characteristic of embryonic radial glial cells (Yuasa, 1996). Tnc^{YFP-low}, CD133⁺ cells respond to Shh and generate forerunners of both astrocytes (Tnc^{YFP-low}, CD15⁺) and of Pax2⁺ cells (Ptf1a⁺). The latter, Pax2-expressing precursors give rise to basket and stellate interneurons and, despite being post-mitotic, accumulate very rapidly during the first postnatal week. It was thought that the vast pools of Pax2⁺ cells generated *de novo* in the PWM stem from a proliferative, Pax2⁻ progenitor (Leto et al., 2009; Weisheit et al., 2006). Our work demonstrated that cells expressing Ptf1a, a factor known for its role in dictating GABAergic fate choice in the cerebellum (Hoshino et al., 2005; Pascual et al., 2007; Yamada et al., 2014), fulfill this function.

Uncovering that Shh signals to primary Tnc^{YFP-low}, CD133⁺ progenitor cells helped explain the molecular foundation supporting PWM-localized neurogenesis. We found following attenuation of Shh signaling in this population during the early neonatal period that a failure to propagate intermediate progenitors of both GABAergic interneurons (Ptf1a⁺ cells and Pax2⁺ cells) and astrocytes (CD15⁺ cells) ensues. We linked this deficit to reduced proliferation within the most mitotically active population we detected occupying the PWM, which also respond to Shh but instead express

transcription factor Sox2, which is known for its function in maintaining NSC status and is itself a putative Shh target gene (Graham et al., 2003; Takanaga et al., 2009). Whether Ascl1-expressing cells, also known to occupy the PWM (Sudarov et al., 2011), fit into the progenitor lineage we have described and similarly respond to Shh is unclear.

A major knowledge gap that persists is what factors influence or determine interneuron subtype specification. Whether Shh may contribute to this process, either by dictating cell cycle dynamics in the PWM niche, a process implicated in interneuron maturation (Leto et al., 2011), or by other means warrants consideration. Both basket and stellate cells emerge from a shared Pax2⁺ precursor pool, but go on to occupy distinct positions within the ML where each establishes a different level of connectivity with PNs. Birth date is thought to be a determinant of laminar placement in the cortex (Leto et al., 2009), and even though basket and stellate cells emerge during overlapping periods, basket cells are positioned along the inner ML, nearest to PN soma and proximal dendrites, while stellate cells reside nearer the pial surface and interface with distal PN dendritic projections (Altman, 1997).

The realization that Shh signaling is activated within the early neonatal PWM led us to uncover that PN-derived Shh promotes GABAergic neurogenesis there by signaling along a novel, inward axis. It seems this 'anterograde' signaling activity may be facilitated by PN axonal projections, in a manner comparable to the fruit fly retina, mouse hair follicle stem cell compartment, and ventral SVZ (Brownell et al., 2011; Huang and Kunes, 1996; Ihrie et al., 2011). PN axons project to targets located deep within the cerebellar core by late embryogenesis (Eisenman et al., 1991; Sillitoe et al., 2009), and therefore infiltrate the PWM concomitant with the onset of Shh expression in PNs (Lewis et al., 2004) and prior to peak basket and stellate production (Sudarov et al., 2011). Theoretically, the proposed infrastructure is present at the appropriate time, but it remains to be determined what factors regulate the targeting of Shh ligand to axons and its dissemination to receiving cells.

A third, and often overlooked, direction of Shh distribution in the nascent and adult cerebellum is laterally to juxtaposed Bergmann glial cells. Although this communication is well documented (Corrales et al., 2004; Lewis et al., 2004), very little is known regarding its significance. Classical studies in chick and mouse originally suggested that Shh is required for inducing Bergmann glial differentiation (Dahmane and Ruiz i Altaba, 1999), yet more recent genetic studies in mice did not reach the same conclusion, indicating instead that maturing Bergmann glia persist in the absence of Shh signaling following Gli2 deletion (Corrales et al., 2006). What factors facilitate distribution of PN-derived Shh to neighboring Bergmann glia, which are often in close physical contact with PN soma and dendrites remain unknown.

Distribution of PN-derived Shh could be regulated at multiple levels, which may have specificity to direction of transportation. Some candidate mechanisms include heparan sulfate proteoglycans that bind the ligand, promoting signal transduction (Chan et al., 2009; Witt et al., 2013), or packaging of Shh ligand into secreted exosomes (Gradilla et al., 2014; Vyas et al., 2014). In *Drosophila*, as well as, in mouse and chicken limb buds, dynamic, actin-based cellular projections called cytonemes, and filopodia, have been shown to facilitate Hh distribution/reception (Bischoff et al., 2013; Sanders et al., 2013). It remains to be determined whether such structures exist on polarized cells like PNs. Dispatched, a 12-pass transmembrane protein that appears to promote secretion and distribution of Shh ligand (Callejo et al., 2011; Ma et al., 2002; Tukachinsky et al., 2012) could also play a vital role in Shh delivery within the nascent and adult cerebellum.

Altogether, our findings support a mechanism that coordinates neurogenesis in the cerebellum to balance the production of excitatory/inhibitory (E/I) neurons. The current understanding of PN function in the mammalian cerebellum has been expanded considerably by our work, which indicates that PNs have a remarkable capacity for regulating developmental events with distinctive temporal and spatial parameters. How related

events are orchestrated in lower vertebrates, such as teleosts, in which Shh signaling is not utilized in the developing cerebellum (Hashimoto and Hibi, 2012), remains unclear. Yet, in the mammalian CNS, PNs, the distinctively central node of the cerebellar system, ultimately manage to assemble a complex neuronal circuit around themselves, which they fine-tune with their own neurotransmission (White et al., 2014). Importantly, this new understanding of PN biology may provide meaningful insight for understanding the etiology of certain neurodevelopmental disorders, such as autism.

Functionally, the cerebellum is known for its essential role in coordinating fine motor control, however, recent studies have revealed a link to higher cognitive function concomitant with the identification of neuronal connections (cerebrocerebellar tracts) between the cerebellum and pre-frontal cortex, the area of the brain most often associated with advanced cognitive function (Suzuki et al., 2012). Interestingly, neuroanatomical findings from postmortem studies of autistic brains revealed loss of PNs (Bauman and Kemper, 2005), and studies in mice have recapitulated autism by conditional disruption of mTOR signaling in PNs, resulting in loss of PNs and significant structural aberrations in surviving PNs (Tsai et al., 2012). Thus, a strong connection seems to be emerging that links PNs with the autism spectrum disorders (AS). Furthermore, E/I imbalance in neurotransmission is an attribute commonly seen in the autistic brain, and numerous possible underlying causes for this have been proposed, including developmental defects (Rubenstein and Merzenich, 2003). Ultimately, the E/I ratio is found to increase, either due to excess excitatory signaling and/or decreased inhibitory signaling.

It is well established that GABAergic signaling plays a prominent role in maintaining inhibitory input to synchronize and balance informational processing, and it has been long thought that suppression of GABAergic inhibitory signaling is a common feature in the autistic brain (Hussman, 2001). Therefore it is important to consider whether underlying cerebellar E/I imbalance could be disruption

of PWM Shh signaling, due to axonal defects or other PN related dysfunction found in the autistic brain. Interestingly, recent human genetic studies have identified deactivating mutations in Patched domain containing-1 (PTCHD1), highlighting it as an autism and/or intellectual disability (ID)-linked gene (Filges et al., 2011; Marshall et al., 2008; Pinto et al., 2010). PTCHD1 encodes a factor with repressive capabilities similar to the Shh receptor Patched (Noor, 2010). *PTCHD1* is highly expressed in the human and mouse cerebellum, where its function is yet to be described. However, it may act as an important negative regulator of Shh pathway activity, and deactivating mutations could result in imbalanced cerebellar neurogenesis and ultimately E/I transmission.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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