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COMMENTARY

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Sonic hedgehog signaling: A conserved mechanism for the expansion of outer radial glia and intermediate progenitor cells and for the growth and folding of the neocortex

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ABSTRACT

The expansion of outer radial glia (oRGs, also called basal RGs) and intermediate progenitor cells (IPCs) has played a key role in the evolutionary expansion and folding of the neocortex, resulting in superior sensorimotor and cognitive abilities. In particular, oRGs, which are critical for both the increased production and lateral dispersion of neurons, are rare in lisencephalic species but vastly expanded in gyrencephalic species. However, the mechanisms that expand oRGs and IPCs are not well understood. We recently identified Sonic hedgehog (Shh) signaling as the first known signaling pathway necessary and sufficient to expand both oRGs and IPCs. Elevated Shh signaling in the embryonic neocortex leads to neocortical expansion and folding with normal cytoarchitecture in otherwise smooth mouse neocortex, whereas the loss of Shh signaling decreases oRGs, IPCs, and neocortical size. We also showed that SHH signaling activity in fetal neocortex is stronger in humans than in mice and that blocking SHH signaling decreases oRGs in human cerebral organoids. Shh signaling may be a conserved mechanism that promotes oRG and IPC expansion, driving neocortical growth and folding in humans and other species. Understanding the mechanisms underlying species-specific differences in Shh signaling activity and how Shh signaling expands oRGs and IPCs will provide insights into the mechanisms of neocortical development and evolution.

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Introduction

The neocortex, a 6-layered structure that computes high-order sensory, motor, and cognitive processes, is both a hallmark and a remarkably divergent part of mammalian brains. Although the layering and thickness of the neocortex remained relatively constant over the course of evolution, its surface expanded dramatically and folded in certain species, resulting in superior sensorimotor and cognitive abilities. Neocortical expansion and folding require 2 coordinated processes that depend on neural progenitors: the increased production of neural cells and their lateral dispersion.[1-4] The primary neural progenitors are radial glia (RGs), whose cell bodies reside in the ventricular zone (VZ) at the apical side of the developing brain and are thus called ventricular RGs (vRGs) or apical RGs (aRGs). vRGs have a radial process that extends to the pial surface and serves as a scaffold for the migration of newborn neurons toward the cortical plate, where later-born neurons settle above early born neurons to form distinct neuronal layers in an inside-out fashion. vRGs produce neurons directly or indirectly via intermediate progenitor cells (IPCs) or outer RGs (oRGs, also called basal RGs) that occupy the subventricular zone (SVZ) basal to the VZ.[5-11] Recent studies suggest that the expansion of oRGs, which not only increases neuron production but also spurs the lateral dispersion of neurons via radial processes of oRGs, plays a critical role in neocortical growth and folding.[12-20] Consistently, oRGs are rare in species with small/smooth brains but are greatly expanded in species with large/folded brains.[10-14,19] Nonetheless, oRGs are present in all the mammalian lineages that have been examined.[14,19,21,22] Furthermore, neocortical folding is prevalent in many mammalian lineages, including marsupials and even egg-laying monotremes (http://neurosciencelibrary.org/index.html). Therefore, mechanisms to induce oRG expansion and neocortical growth and folding appear to have been conserved from a common

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ancestor of mammals but selectively fortified or inactivated in different lineages, giving rise to large/folded or small/smooth brains.[14,19,21-23] Recently, we provided evidence indicating that Sonic hedgehog (Shh) signaling has been central to these mechanisms[24] (Fig. 1).

Shh signaling expands oRGs and IPCs, leading to neocortical growth and folding in mice

Shh signaling is a conserved mechanism that regulates many aspects of animal development. Notably, mutations that attenuate SHH signaling cause microcephaly in humans,[25-27] suggesting that SHH signaling regulates brain size in humans. In mice, the loss of Shh signaling in the neocortex decreases its size;[28] however, the gain of Shh signaling did not increase the neocortical size but disrupted the patterning and specification of neural progenitors.[29-32] To study the role of Shh signaling in neocortical development beyond patterning and specification, we conditionally removed or activated Smoothened (Smo, an activator of Shh signaling) by using *GFAP::Cre*, which induces recombination at embryonic day 13.5, when the patterning and specification of neural progenitors has already been established. The expression of a constitutively active form of Smo (SmoM2) in vRGs and their progenies in *GFAP::Cre; SmoM2*$^{fl/+}$ (*SmoM2* mutant) mice significantly increased the size of the neocortex. Remarkably, SmoM2 induced folding in the cingulate cortex without affecting the normal cytoarchitecture. As in the folded brains of larger mammals, in which upper-layer (layer II and III) neurons are much more expanded than are deeper-layer neurons and the white

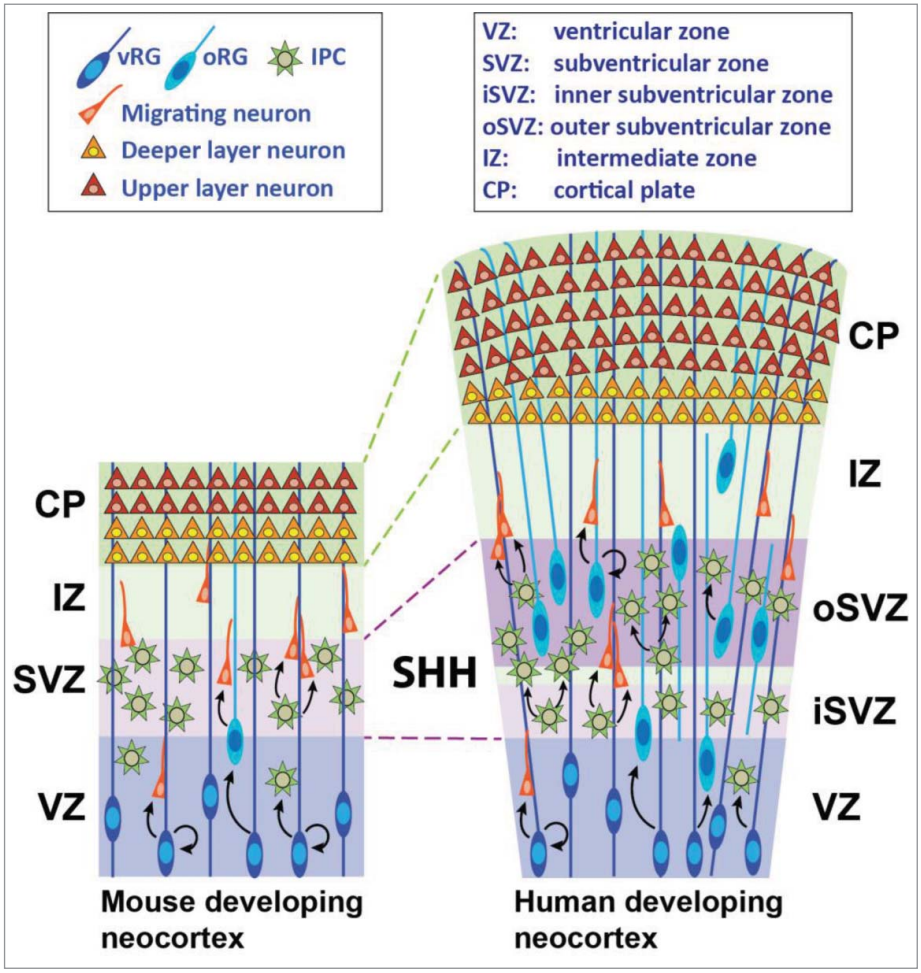

Figure 1. In species with a large and folded neocortex, oRGs and IPCs are expanded in the cortical SVZ, which is divided into the inner and outer SVZs (iSVZ and oSVZ). SHH promotes this expansion, leading to neocortical growth and folding. Mechanistically, SHH expands oRGs by increasing their self-renewal and production from vRGs and expands IPCs by increasing their self-amplifying divisions in the SVZ. *Shh* in mice and *SHH* in humans are highly expressed in the VZ of the ventral forebrain, suggesting trans-ventricular delivery of SHH proteins to the neocortex.

matter extends into the gyri, upper-layer neurons were specifically increased in the folded cingulate cortex of *SmoM2* mutants, and the corpus callosum was extended into the folded area. Upper-layer neurons were not increased in the lateral part of the neocortex that did not show folding, suggesting that increased upper-layer neurons induced neocortical folding. The medial-to-lateral gradient of the upper-layer neuron increase reflected the expression pattern of SmoM2 in *GFAP::Cre; SmoM2*$^{fl/+}$ mutants. The expression of SmoM2 in *Nestin::Cre; SmoM2*$^{fl/+}$ or *Nestin::CreER; SmoM2*$^{fl/+}$ mice, which do not show such a SmoM2 expression gradient, induced folding outside the cingulate cortex too. Therefore, the mechanism that underlies neocortical folding in *SmoM2* mutants must be a general one rather than being specific to the cingulate cortex.

To understand the cellular mechanism by which SmoM2 expanded upper-layer neurons, we investigated whether and how SmoM2 changed the number and behavior of neural progenitors. The number of vRGs was not changed, but the numbers of oRGs and IPCs were greatly expanded in *SmoM2* mutants. Notably, SmoM2 expanded oRGs and IPCs via distinct mechanisms by affecting the behavior of all 3 types of progenitor. SmoM2 did not affect the proliferation rate of oRGs or vRGs, but it increased self-renewal of oRGs and changed the vRG division modes to produce more oRGs and fewer IPCs and neurons. vRGs dividing on an axis horizontal to the ventricular surface mostly produce neurons or IPCs, whereas those dividing nonhorizontally produce oRGs.[11,33] Nonhorizontal divisions were markedly increased in *SmoM2* mutants, as compared to controls. Thus, SmoM2 expanded oRGs by promoting their initial generation from vRGs and their subsequent self-renewal. In contrast, SmoM2 decreased the generation of IPCs from vRGs but increased their proliferation and self-amplifying divisions, leading to their great expansion in the SVZ. Similarly, IPCs of primates divide to make more IPCs before producing neurons,[12,34] whereas IPCs of mice and rats mainly divide just once to produce 2 neurons.[6-8]

Consistent with the results of gain-of-function experiments, we found that endogenous Shh signaling is required to expand oRGs, IPCs, upper-layer neurons, and the neocortex. The loss of Shh signaling in *GFAP::Cre; Smo*$^{fl/fl}$ mutants caused phenotypes opposite to those of *SmoM2* mutants. Compared to wild-type mice, the *GFAP::Cre; Smo*$^{fl/fl}$ mice had abnormally small brains with fewer upper-layer neurons, significantly fewer oRGs and IPCs (but a similar number of vRGs), and a decreased proportion of vRGs dividing nonhorizontally. Taken together, these findings show that Shh signaling promotes key developmental characteristics of large and folded brains, namely oRG expansion and self-amplifying IPC division, which a comparative study of 102 mammalian brains proposed to be necessary and sufficient for the evolution of an expanded and folded neocortex.[35]

Shh signaling is required for human oRG expansion

Based on our mouse study, we predicted that Shh signaling activity would correlate with the number of oRGs and IPCs and be stronger in gyrencephalic species than in lisenscephalic species. Indeed, by comparing RNAseq data and the results of *in situ* hybridization experiments, we found that SHH signaling activity is stronger in human fetal neocortex than in mouse embryonic neocortex. Furthermore, the developmental change in SHH signaling activity correlated with oRG expansion in human fetal cortex. In mice, the regional difference in Shh signaling activity in the neocortex correlated with the number of oRGs. A previous study in ferrets showed that Shh signaling activity is significantly higher in the VZ area that gives rise to the thick SVZ containing many oRGs than in the VZ area that gives rise to the thin SVZ containing fewer oRGs.[36]

To functionally test whether SHH signaling expanded human oRGs and IPCs, we employed human cerebral organoids that recapitulate key features of the developing human cortex, including abundant oRGs.[37-41] In contrast to mouse vRGs, but similar to human vRGs in slice culture,[33] more than half of the vRGs in the organoids divided nonhorizontally. SANT1 (a Smo inhibitor) strongly decreased the incidence of nonhorizontal division, similar to the low incidence of nonhorizontal division in mouse vRGs, and subsequently decreased the number of oRG-like cells outside the VZ, whereas neither effect was seen with SAG (a Smo agonist). Accordingly, we showed that SHH signaling was intrinsically active in the organoids and could be blocked by SANT1 but could not be further increased by SAG. The number of IPCs was

very low and was not significantly affected by SANT1 or SAG. These results suggest that Shh signaling promotes oRG expansion in gyrencephalic species.

Conclusion and future directions

Our study showed that Shh signaling promotes oRG and IPC expansion, leading to neocortical growth and folding. Shh signaling is the first signaling pathway with these properties to be identified. This role of SHH signaling appears to be conserved, at least in mice and humans. SHH signaling activity is stronger in human fetal cortex than in mouse embryonic cortex and correlates with the number of oRGs in both species, suggesting that Shh signaling may have played important roles in the evolutionary growth and folding of the neocortex. These findings linking Shh signaling with oRG and IPC expansion, neocortical growth, and evolution raise important questions: what are the mechanisms underlying the difference in Shh signaling activity in the developing neocortex of humans and mice; what are the molecular mechanisms by which Shh signaling differentially affects 3 different neural progenitor types; and are these mechanisms conserved? The answers to these questions will provide fundamental insights into the development and evolution of mammalian brains.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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