Arteriovenous malformations: the newest Sonic hedgehog game in the postnatal brain

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The morphogen Sonic hedgehog (Shh) is crucial for the embryonic development of the central nervous system, but is also an important player in the postnatal brain, with activities that range from the modulation of self-renewal and specification of neural stem cells to the regulation of neural regeneration after injury. Active Shh signaling occurs also in molecular subclasses of brain tumors, such as medulloblastoma and adult glioma. In some cases, Shh-responsive cells may even be the brain tumor cells of origin. We have recently identified a novel possible role for Shh in the post-natal brain, which is a contribution to the pathogenesis of arteviovenous malformations (AVMs). Brain AVMs are abnormal tangles of vessels which directly shunt blood from the arterial to the venous circulation without an interposed capillary bed. They are an important cause of intracranial hemorrhage and account for about 50% of strokes in childhood. Despite intense investigation, etiology and pathogenesis of brain AVMs remain poorly understood and this hinders the development of effective therapeutic strategies. In this article, we summarize the results of our recent study, in which we demonstrated that the Shh signaling pathway is aberrantly active in the endothelium of human brain AVMs and that stereotactic injection of Shh in the rat brain results in an angiogenic response that displays many of the features that are typical of AVMs, such as the presence of dilated, tortuous, and entangled arterial and venous vessels interconnected to each other by direct arteriovenous shunts. In this article, we also discuss the multiple mechanisms potentially responsible for the upregulation of the Shh pathway in AVMs and those through which Shh might contribute to the pathogenesis of AVMs, including abnormal angiogenesis, alterations of the blood-brain barrier, and promotion of survival and inhibition of apoptosis of neural and endothelial cells. Finally, we discuss the clinical and therapeutic implications of a potential involvement of the Shh pathway in brain AVM pathobiology, including the possibility to use anti-Shh molecules for the treatment of brain AVMs in humans.

Shh, initially known for its crucial role during the embryonic development of the central nervous system (CNS), is now recognized as an important and active player in the postnatal brain, with pleiotropic activities that range from the modulation of the self-renewal and specification of neural stem cells (NSCs) to the regulation of post-injury neural regeneration. There is a large body of evidence demonstrating the importance of the Shh signaling pathway in the adult CNS, generated in a number of experimental models. For instance, it is known that Shh is upregulated in the zebrafish floor plate after spinal cord transection and that treatment with cyclopamine – an inhibitor of the pathway – reduces the number of cells expressing markers of motor neuron regeneration (Reimer et al., 2009; Belgacem et al., 2016). It is also known that Shh is upregulated in facial motor neurons after facial nerve axotomy in adult rats and that treatment with cyclopamine leads to decreased number of surviving motor neurons after injury (Akazawa et al., 2004; Belgacem et al., 2016). In mice, enhancing Shh signaling after ischemic stroke improves functional recovery (Chechneva et al., 2014; Belgacem et al., 2016) and eliminating Shh signaling is detrimental for oligodendrocyte differentiation and remyelination (Samanta et al., 2015; Belgacem et al., 2016). Taken together, these findings suggest that, similarly to development, appropriate regeneration in the adult nervous system may require recapitulation of the Shh signaling. However, it is not always clear whether the reactivation of Shh signaling in the adult brain is beneficial. Indeed, there are studies that link Shh upregulation to aberrant and detrimental responses within the CNS and even to cancer. Indeed, active Shh signaling occurs in stem-like brain tumor cells and molecular subclasses of brain tumors, including pediatric medulloblastoma and juvenile and adult glioma (Alvarez-Buylla and Ihrie, 2014). In some cases, a Shh-responsive cell may also be the brain tumor cell of origin (Alvarez-Buylla and Ihrie, 2014). Therefore, Shh is likely an important mitogen, or self-renewal factor, not only under normal conditions, but also during oncogenesis in the postnatal brain.

Shh and brain AVMs: Recently, we have identified a novel role for the Shh signaling pathway in the adult brain, i.e. a possible involvement in the pathogenesis of brain AVMs (Giarretta et al., 2020). Brain AVMs consist of abnormal tangles of small vessels (called *nidi*) which directly shunt blood from the arterial to the venous circulation without an interposed capillary bed. They are an important cause of intracranial hemorrhage in younger persons and account for 1–2% of all strokes in the general population and 50% of strokes in childhood (Chen et al., 2014). Whether brain AVMs are congenital lesions or develop *de novo* in postnatal life is still controversial. A review of all reported cases of *de novo* AVM formation was published in 2018 (Dalton et al., 2018). The authors found 29 cases of brain AVMs in subjects who did not have any lesion in prior neuroimaging studies (such as *Magnetic Resonance Imaging and Digital Subtraction Angiography*). Thus, it is possible to hypothesize that brain AVMs may both arise in the embryo or form in childhood or even adulthood, perhaps through the postnatal dysregulated activation of developmental processes. However, despite intense investigations, etiology and pathogenesis of brain AVMs remain poorly understood and this hinders the development of effective therapeutic strategies.

In our recent study, we found aberrant expression of Shh in the endothelial layer of human brain AVMs, by immunohistochemical analyses. In the same endothelial layers, we also found aberrant expression of Gli1 and COUP-TFII, which respectively are the main transcription factor of the Shh canonical pathway and a target of the non-canonical Shh signaling (Giarretta et al., 2020). This is of note, because it is known that *nidi* of brain AVMs are made up of not terminally differentiated vessels that co-express both arterial and venous markers (Thomas et al., 2018) and Shh is an important regulator of arterial versus venous identity of endothelial cells (ECs) (Corada et al., 2014). Indeed, it is well established that Shh regulates the vascular endothelial growth factor /Notch pathway to induce arterial differentiation of ECs (Lawson et al., 2002; Swift and Weinstein, 2009), but also has the ability to activate COUP-TFII, which is a down-regulator of Notch and is specifically expressed in venous, but not arterial, ECs (You et al., 2005). Since vessels of normal human brain are negative for Shh expression (Giarretta et al., 2020), it is possible to hypothesize that the Shh signaling plays a role in the pathogenesis of brain AVMs. We tested this hypothesis by injecting a plasmid carrying the Shh gene in the brain of rats and demonstrating that focal intracerebral Shh upregulation induces the growth of tangles of vessels that display many characteristics that are reminiscent of human brain AVMs. Indeed, Shh-induced cerebral neovessels are enlarged and tortuous, display both venous and arterial phenotypes, and are interconnected by direct arteriovenous shunts without an interposed capillary bed (Giarretta et al., 2020).

Based on these findings, one might speculate that abnormal upregulation of the Shh signaling in a specific brain region might lead to AVM formation, perhaps in subjects

with a favorable background. This would be consistent with the more general concept that postnatal reactivation of developmental angiogenic pathways might play a critical role in the generation of brain AVMs in the adult. It would also be consistent with the so-called 'response-to-injury' paradigm, which proposes that, in the presence of an underlying structural defect, an inciting event might lead to an abnormal response, with the eventual generation of a brain AVM (Lawton et al., 2015). This might occur through multiple potential mechanisms. A possible one is the stimulation of angiogenesis, based on the notion that Shh has the ability to stimulate various families of angiogenic growth factors (Pola et al., 2001). Another possible mechanism is an alteration of the blood-brain barrier, since the Shh pathway is an important regulator of the blood-brain barrier integrity (Alvarez et al., 2011). Therefore, aberrant upregulation of Shh might affect vessel permeability, which is a feature of brain AVMs (Zhang et al., 2016). It has also been demonstrated that Shh expression confers a survival advantage to neuronal cells during the response to excitotoxic insults, compared to cells that do not express Shh (Gonzalez-Reyes et al., 2012, 2019). The ability of Shh to promote survival and inhibit apoptosis has been demonstrated also in ECs (Chinchilla et al., 2010) and this might be relevant for brain AVM formation and growth.

Certainly, it remains to be clarified which inciting events might result in aberrant reactivation of the Shh pathway in the brain. Based on the knowledge that we have of this pathway, we may speculate that these might be hypoxia, ischemia, inflammation, microscopic traumas, infections, or mechanical injuries. Among these, neuroinflammation is a documented trigger of Shh upregulation in the brain. Indeed, Shh transcription is induced in astrocytes upon exposure of these cells to inflammatory cytokines and Gli1 is upregulated in the inflammatory peri-ischemic area in the early stage of stroke (Amankulor et al., 2009; Giarretta et al., 2019). Regarding other potential inciting factors, it is known that neural cells increase *Shh* mRNA levels under hypoxic conditions and that Shh is secreted by activated astrocytes when these cells are incubated under oxygen-glucose deprivation conditions or are exposed to oxidative stress (Dai et al., 2011; He et al., 2013; Giarretta et al., 2019).

Also, it remains to be elucidated which structural pre-existing defects might constitute a favorable background for an aberrant angiogenic response to the above-mentioned inciting events. A possibility is the presence of a genetic influence. For instance, it is known that mutations in the endoglin, activin-like kinase 1 (ALK1, or ACVLR1), and SMAD4 genes are involved in the pathogenesis of hereditary hemorrhagic telangiectasia (HHT), a disease that is often characterized by the presence of brain AVMs. It is also known that somatic activating mutations of KRAS (Nikolaev et al., 2018) or BRAF (Goss et al., 2019), two pro-oncogenic genes, may be detected in the ECs of sporadic (non-HHT-related) brain AVMs. These mutations have been associated with increased expression of angiogenic genes and stimulation of the Notch signaling pathway, further supporting the hypothesis that certain genetic backgrounds may make individual subjects more prone to develop AVMs. It has also been hypothesized that variations in genes encoding for inflammatory cytokines and angiogenic factors might contribute to AVM pathogenesis by enhancing or maintaining a pro-inflammatory and/or pro-angiogenic state that favors lesion formation (Mouchtouris et al., 2015). A practical example of the interaction between an inciting factor and a favorable background has been provided by Hao et al., who have shown that injection of vascular endothelial growth factor into the brain of mice with heterozygous deletion of ALK1 results in the formation of vascular lesions that resemble brain AVMs (Hao et al., 2010). Importantly, although being characterized by the activation of potential pro-oncogenic pathways such as KRAS, BRAF, or Shh, brain AVMs are not associated with cancer, suggesting a context-dependent role for these mediators in the endothelium. Also, it should be noted that, although dysregulated activation of the Shh pathway has been implicated in the pathogenesis of many cancer types, including brain tumors, at the moment there is no evidence in the literature that chronic administration of Shh agonists has led to the development of cancer.

The proposed hypothetical mechanism through which Shh may contribute to brain AVM formation is schematically presented in **Figure 1**. However, this is certainly a simplification. Indeed, to fully understand the role played by the Shh pathway in AVM pathogenesis, a number of issues still need to be addressed. First, it should be clearly determined which physiological and/or pathological conditions have the ability to induce upregulation of the Shh pathway in specific brain regions. Second, it should be clarified which cells in the brain produce Shh in response to a given inciting factor and which cells respond to such endogenous production of Shh. Third, the molecular effects induced by Shh in responding cells should be precisely investigated. These effects might be different from cell to cell and might also depend on the Shh gradient to which cells are exposed. There is indeed evidence that Shh may promote either cell proliferation or cell apoptosis depending on its concentration, with an inverted "U-shaped" dose-effect (Daynac et al., 2016). Also, it should be studied whether inhibition of the Shh pathway may have beneficial therapeutic effects in experimental models of brain AVMs. Finally, it should be highlighted that much of the information that we have on the pathogenesis of brain AVMs derives from animal models, which are never able to fully recapitulate the human disease. Therefore, additional studies are needed before being able to translate these findings to humans.

Perspectives and outlooks: At the moment, it is no clear what is the most effective treatment for brain AVMs. Even more importantly, there is no evidence that any treatment is better than simple medical management. Indeed, in 2014, the ARUBA trial has demonstrated that medical management alone is superior to interventional therapy for the prevention of death or stroke in patients with unruptured brain AVMs. It is important to note that in the ARUBA study, more than 10% of patients in the medical management group and more than 30% of patients in the interventional therapy group experienced a stroke or died, which indicates that the prevalence of adverse events is high in this population and there is the urgent need of more effective treatment options (Mohr et al., 2014). In this scenario, new exciting therapeutic opportunities might come if it will be confirmed that the Shh pathway is an important player in the pathogenesis of brain AVMs. Indeed, inhibitors of the Shh pathway are already available and some of them have been approved by the Food and Drug Administration for the treatment of diseases, such as basal cell carcinoma and acute promyelocytic leukemia. Anti-Shh strategies are currently under investigation for the treatment of other neoplastic diseases, such as medulloblastoma, small cell lung cancer, pancreatic cancer, intracranial meningioma, recurrent glioblastoma, prostate cancer, renal cell carcinoma, and colon cancer. A future perspective would be to use systemic administration of anti-Shh molecules in association with surgical procedures, or to combine local delivery of anti-Shh drugs with endovascular selective occlusion of AVM feeders.

Conclusions: We have found that AVMs are a novel playground for Shh in the brain. Elucidating mechanisms and factors regulating Shh upregulation in the adult brain will increase our understanding of the role played by Shh in AVM lesion formation, with the possibility to design therapeutic strategies that interfere with the natural progression of the disease. A better comprehension of the pathophysiology of brain AVMs will also have important implications in other areas of neurovascular biology and neural regeneration and disease.

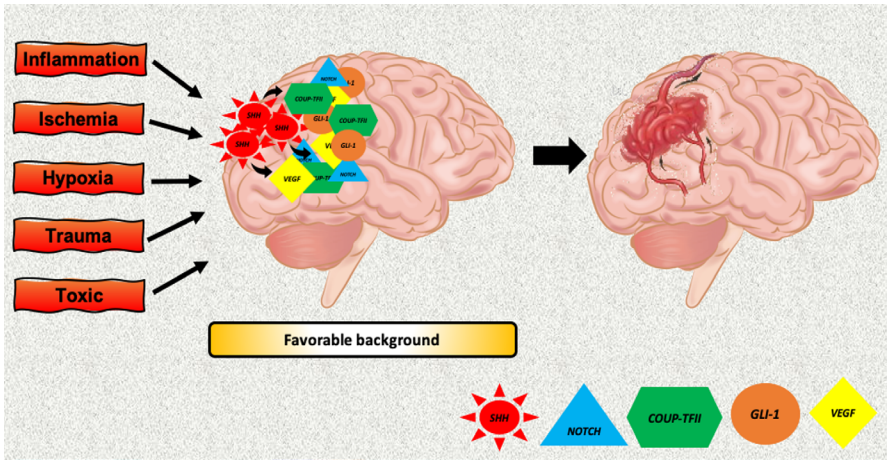

Figure 1 | **Proposed mechanism of Shh contribution to the development of arteriovenous malformations in the brain.**
In the presence of a genetically or anatomically favorable background, inciting events, such as inflammation, ischemia, hypoxia, or traumatic and toxic injuries, may lead to the upregulation of Shh in a specific brain area, with the activation of the Gli1-dependent canonical pathway, which leads to the upregulation of the VEGF/Notch signaling. Shh upregulation also leads to the activation of COUP-TFII via a non-canonical pathway. The effect of the concomitant activation of Notch and COUP-TFII is the generation of a brain arteriovenous malformation. VEGF: Vascular endothelial growth factor; Shh: Sonic hedgehog.

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