Actual Biol 38 (105): 197-209, 2016 | **DOI: 10.17533/udea.acbi.v38n105a07**

Sonic Hedgehog (SHH) pathway in the adult brain: key signaling for astrocyte reactivation and brain repair

Señalización Sonic Hedgehog (SHH) en el cerebro adulto: vía crucial para la reactivación de los astrocitos y la reparación del cerebro

Olga M. Bermúdez-Muñoz[1]

Abstract

While neurons play a key role in neurotransmission in the nervous central system **(CNS)** of animals, glial cells are crucial for neuron support and brain maintenance. Recent studies reveal that glial cells regulate the release and reuptake of neurotransmitters, pyruvate and glutathione metabolism, ion buffering, the organization of blood brain barrier and ensures the production of myelin and cerebrospinal fluid. The activity of glial cells is coordinated by the communication between neurons and the glia. Among cell signals in the brain, Sonic Hedgehog **(SHH)** pathway plays a key role regulating the development and the patterning of the central nervous system. In the adult brain, SHH has been found to be secreted by neurons and astrocytes, and to regulate in this manner, neuro-glial interactions. Upon brain injury, SHH signaling appears to be (re)-activated in the adult brain and may be related with tissue regeneration. The glial cells and more particularly astrocytes are key cells responding to brain injury and participating in brain repair. Interestingly, astrocyte response is mediated by SHH activation in these cells that elicits diverse cell reactions in the brain leading to neuroprotection and reinforcement of the blood brain barrier upon injury. This review highlights the important role of glial cells and more specifically of astrocytes in brain physiology, the implication of SHH signaling in brain organization and function, and finally, how SHH signaling regulates astrocyte re-activation and cell response to tissue injury and repair in the brain in the adult organism.

Key words: astrocyte, brain injury, glia, Hedgehog signaling, tissue repair

Resumen

Mientras que las neuronas juegan un papel fundamental en la neurotransmisión en el sistema nervioso central de los animales, las células gliales son cruciales para dar sostén a las neuronas y por lo tanto, para el funcionamiento del cerebro. Estudios recientes han puesto de manifiesto que las células gliales regulan la liberación y reciclaje de neurotransmisores, el metabolismo del piruvato y del glutatión, sirviendo de tampón para diferentes iones, participando en la organización de la barrera hematoencefálica y en la producción de mielina y del líquido cefalorraquídeo. La actividad de las células gliales se encuentra estrechamente coordinada por la comunicación entre las neuronas y la glía. Entre la señalización celular del cerebro, la vía Sonic Hedgehog **(SHH)** juega un papel importante al regular el desarrollo y patrón del sistema nervioso central. En el cerebro adulto, la proteína SHH es secretada por las neuronas y por los astrocitos y media de esa manera las interacciones neuro-gliales. Cuando ocurre un daño en el cerebro, la vía de señalización SHH es (re)-activada en el cerebro adulto. Las células gliales y particularmente los astrocitos, son células esenciales para la respuesta del cerebro frente a un daño y para su reparación. La respuesta de los astrocitos se encuentra mediada por la activación de la vía SHH en estas células. En este artículo se revisa la importancia de las células gliales y específicamente de los astrocitos en la fisiología del cerebro, la implicación de la vía de señalización SHH en la organización y funcionamiento del cerebro, y cómo la señalización SHH regula la re-activación de los astrocitos y la respuesta celular frente al daño tisular y a la reparación del cerebro en el organismo adulto.

Palabras clave: astrocito, daño cerebral, glía, reparación tisular, señalización Hedgehog

Received: March 2016; accepted: May 2016 *(Recibido: marzo 2016; aceptado: mayo 2016)*.
[1] Docente. Grupo de Investigación Genética, Regeneración y Cáncer, Instituto de Biología, Universidad de Antioquia. A. A. 1226. Medellín (Antioquia), Colombia. Email: <olga.bermudez@udea.edu.co>

INTRODUCTION

While neurons play a key role in neurotransmission in the central nervous system **(CNS),** glial cells are crucial for neuron support and brain maintenance. The term neuroglia was initially used by the German anatomist Rudolf Virchow to describe non-neuronal cells that constitute scaffolding substance in the brain (Rudolf Virchow 1862). Indeed, glia cells were initially considered glue cells between the neurons and the nerves, and thus passive actors in brain physiology. Nonetheless, recent findings have uncovered the active role of glia cells during brain development and brain function in the adult. According to their origins during brain development, two classes of glial cells are distinguished: macroglia derived from ectoderm and microglia cells that come from monocyte-macrophage lineage (Zuchero and Barres 2015). Microglia are specific immune cells representing the resident macrophages in the CNS activated upon injury that are able to stimulate the immune cells and to phagocytose damaged neurons (Chan et al. 2007). On the other side, macroglia corresponds to the most abundant and more heterogeneous population of glial cells in the brain, able to accomplish a wide variety of functions, critical for brain function. Macroglia cells can give rise to neurons *in vitro* and *in vivo* and thus represent very interesting cells for research in brain repair upon injury and in neurodegenerative diseases (Barres 1999, Dimou and Götz 2014). Glia cells are also essential for the guidance and stabilization of axons in neurons, establishing in this manner a precise and complex neuronal connection network in the developing and adult CNS (Chotard and Salecker 2004). Additionally, glia cells regulate neuronal synapse formation and length, and some glia cells have receptors for neurotransmitters, being able to modulate neuronal activity and synaptic plasticity in both physiological and pathological conditions (Allen 2013, Ben Achour and Pascual 2010, Eroglu and Barres 2010, Haydon 2001). Besides the role of glia cells on the regulation of neuron synapses, glial cells are fundamental for the capture of energy compounds from the blood and for its delivery to neurons, playing an important role in glucose metabolism (Tabernero et al. 2006, Yang et al. 2013). Finally, glial cells are required for the formation of the blood brain barrier **(BBB),** a specific barrier in the brain that preserves a regulated microenvironment for reliable neuronal signaling. Although this barrier is lined by endothelial cells, the interplay of these cells with neurons and glial cells is crucial for regulating BBB function (Alvarez et al. 2013, Cheslow and Alvarez 2016, Prat et al. 2001). Overall, macroglia cells play key roles in brain development (Campbell and Götz 2002), brain metabolism (Tabernero et al. 2006), synapse plasticity (Allen 2013, Ben Achour and Pascual 2010, Eroglu and Barres 2010, Eshed-Eisenbach and Peles 2013), axon guidance (Chotard and Salecker 2004, White and Krämer-Albers 2014), brain barrier formation (Elsayed and Magistretti 2015, Ransom et al. 2003), neuron generation (Barres 1999, Dimou and Götz 2014) and cell signaling in physiological and pathological conditions (Campbell and Götz 2002, Fields and Burnstock 2006, Milligan and Watkins 2009, Nagelhus et al. 2013, Ransom et al. 2003).

Due to the key functions that glial cells perform in the brain, deregulation in these types of cells is associated with changes in brain function related with ageing, CNS body regulation and neurological diseases. *In vitro,* preclinical and clinical studies point for glial abnormalities in psychiatric disorders and neurological diseases including Amyotrophic lateral sclerosis, Alzheimer´s disease and Major depression disorder (Elsayed and Magistretti 2015, Schitine et al. 2015; Verkhratsky et al. 2014). Glial cells and more specifically macroglia represent then a very important cell population in the brain that far from being only "glue material" for the neurons, accomplish different actions that regulate neuron and brain function. These diverse functions are accomplished by specialized macroglia cells. While ependymal cells are essential for cerebrospinal fluid production, oligodendrocytes are necessary for myelin synthesis. NG2 (NG2 gene codes for chondroitin sulfate proteoglycan 4, CSPG4) positive cells represent a more recently identified glial cells, acting as progenitor cells in the adult mammalian brain and possibly having other functions that remain to be uncovered in the future. Among the macroglia cells, astrocytes represent the more abundant and heterogeneous group of cells, having different functions such as regulating the release and reuptake of neurotransmitters, pyruvate and glutathione metabolism, ion buffering and organization of the blood brain barrier. The diversity of astrocyte functions may be related with differences in cell development and differentiation that originate different astrocyte types (Bayraktar et al. 2015, Chaboub and Deneen 2012, Khakh and Sofroniew 2015, Schitine et al. 2015). Morphologically, two main types of astrocytes have been described in cerebral cortex: protoplasmic and fibrous astrocytes. Protoplasmic astrocytes have highly branched processes, are extensively distributed in the gray matter and envelop blood vessels, forming the outer wall of the blood brain barrier. On the other hand, fibrous astrocytes exhibit long processes located in the white matter, having a star-like appearance. If this traditional classification exposed some differences between astrocyte populations,

molecular analysis have revealed the considerable diversity of astrocyte cells (Hochstim et al. 2008, Miller and Szigeti 1991, Okano-Uchida et al. 2004, Ståhlberg et al. 2011).

At the molecular level, astrocytes can be characterized by the expression of glial fibrillary acidic protein **(GFAP)**, calcium-binding protein S100B, and glutamate-aspartate transporter and glutamate transporter 1 **(GLT-1)**. Although the expression of these proteins was for long time used as a specific characterization of astrocytes, the unbiased integrative analysis of different astrocyte-rich cultures and CNS tissues revealed that there is a larger set of astrocyte-specific genes, that includes 85 human genes (Bachoo et al. 2004). These results demonstrate that there is a wider set of specific expressed genes in astrocyte cells and that some of these genes are expressed by specific astrocyte subgroups. Recent gene transfer techniques, genetically modified mice and single cell analyses have shown that astrocytes can also differ in their embryonic origins, in calcium signaling and cell metabolism, and that the brain can exhibit a regional and temporal heterogeneity in its astrocyte composition (Bayraktar et al. 2015, Chaboub and Deneen 2012, Hochstim et al. 2008, Khakh and Sofroniew 2015, Miller and Szigeti 1991, Miyamura et al. 1998, Ståhlberg et al. 2011, Tabata 2015). Due to the fact that astrocytes represent an important cell type in the CNS, accomplishing different functions related with neuron growth, differentiation, metabolism, and signaling, we consider that these glial cells may play a crucial role in the regulation of the brain parenchyma and the neurovascular unit in physiological and pathological conditions.

The coordination of cell function in the brain is accomplished thanks to a regulated signaling network. Among signaling pathways that enable the communication between neuron and cells, Hedgehog cascade plays a crucial role in brain formation but also in the adult brain, facilitating cell-cell interactions. Interestingly, Hedgehog signaling is active in glial cells during brain development and in the adult stage, in precursor cells (Han et al. 2008, Palma et al. 2005). Given the fact that brain repair upon injury is critical to ensure brain function, we aimed to investigate if glial cells and in particular astrocytes elicit a signaling reaction that regulates brain response. Considering that brain repair upon injury may recapitulate aspects of brain formation and thus of brain development, we hypothesize that the Hedgehog signaling pathway, activated during brain formation and in glial precursor cells, might be involved in brain response to injuries. In order to assess this question, we reviewed on one hand the importance of Hedgehog signaling in astrocyte formation and function, and on the other hand, evidences that Hedgehog pathway is implicated in brain response upon injury, through astrocyte activation.

MATERIALS AND METHODS

The review is based on international published articles, available on the Pubmed database of the National Center of Biotechnology Information (<www.ncbi.nlm.nih.gov>). This database was chosen because it offers an updated and wide set of journals of Cell Biology, Molecular Biology, Cell Signaling and Biomedical Sciences, main fields of interests in this investigation. The search was made using the keywords "hedgehog" and "brain" and "glioma", from the start dates of the database to the 31th of January of 2016. Literature was reviewed in order to test the hypothesis that Hedgehog signaling plays an important role in glial cells and moreover, in astrocyte response upon brain injury. Confident and reproducible published information was selected to assess the working hypothesis.

Hedgehog pathway: a key signaling for astrocyte development and function. Hedgehog **(HH)** signaling pathway is a key signaling cascade for the development and patterning of the (CNS). HH ligands act as morphogens, having the capacity to enhance cell responses according to gradient concentrations, at short and long-range distances (up to 300 μm in the limb bud of vertebrates) (Briscoe and Thérond 2013). Canonical Hedgehog signaling begins with the secretion of the ligand HH. Once synthesized, HH suffers different post-translational modifications that ensure its secretion and signaling properties. First, the N-terminal signal sequence residues are removed from HH protein. Then, in the Endoplasmic Reticulum **(ER),** palmitate is added to the N-terminal extremity of SHH, increasing the hydrophobicity of the molecule and its secretion by shedding. Although nonpalmitoylated HH has been found to be functional, it has less signaling activity than palmitoylated forms, *in vitro* and *in vivo* (Guerrero and Kornberg 2014). Also in the ER, HH undergoes autoproteolytic cleaveage, generating one N-terminal fragment containing a Hedge domain, linked to cholesterol and that has signaling function. The autocleavage of HH also produces a C-terminal polypeptide, containing a Hog domain which promotes the autocleavage reaction, that is degraded in the ER by the ERAD (ER-associated degradation) cascade (Ingham et al. 2011). The fact that non-cholesterolyated HH has decreased signaling capacities and do not exhibit normal distribution in the tissues, points for a role of cholesterol in HH secretion and gradient formation (Guerrero and Kornberg 2014).

Thus, HH lipid modification appears to play an important role for regulated HH secretion and distribution in the tissue. HH signaling in the brain during development and brain injury might then be facilitated upon secretion of lipid modified HH ligand. Evolutionarily, the apparition of Hedgehog proteins may be related with Hedge and Hog domain ancestors. While the Hedge domain has been found in proteins of *Streptomyces albus, Monosiga* spp., in the metazoan Amphimedon queenslandica and in the cnidarian *Nematostella vectensis,* the Hog domain has been reported in red algae, dinoflagellates, mosses, and metazoan. Hedgehog proteins may have arisen more than 650 million years ago by the combination of Hedge and Hog domains, in the common ancestors of Cnidarians and bilateral organisms (Ingham et al. 2011, Matus et al. 2008).

While in invertebrates there is only one HH ligand, in vertebrates there are three: Sonic Hedgehog **(SHH)**, Desert Hedgehog **(DHH)**, and Indian Hedgehog **(IHH)**. Evolutionary, DHH is more closely related with *Drosophila* HH. In vertebrates, DHH has been reported to be mainly expressed in gonads, IHH in bone and SHH appears as the most broadly expressed HH ligand in the organism. In the brain, SHH has been the consistently found HH isoform in the brain and will be then considered as the HH ligand of interest in the following sections. Once secreted by the producing cell, SHH is received by specific twelve transmembrane proteins Patched **(PTCH)** (Robbins et al. 2012). Some proteins like Interference Hedgehog **(IHOG)** and brother of IHOG **(BOI)** in *Drosophila melanogaster* and their orthologs in vertebrates Cell adhesion molecule Downregulated by Oncogenes **(CDO)** and brother of CDO **(BOC)**, and also growth arrest-specific 1 **(GAS1)** act as co-receptors for HH proteins (Robbins et al. 2012). Interestingly, in the absence of HH ligands, PTCH receptors do not activate HH pathway but rather inhibits the protein Smoothened **(SMO)**, which is essential for intracellular transduction. HH ligands release the repression of PTCH on the seven transmembrane G protein coupled receptors SMO. Although the precise mechanism by which PTCH represses SMO activity is not understood, it may be related with oxysterols, products of cholesterol oxidation, transported across the membrane by PTCH. Oxysterols have been found to regulate SMO activity and to increase Gli-mediated Hedgehog signaling activation (Briscoe and Thérond 2013, Gorojankina 2016, Nedelcu et al. 2013, Robbins et al. 2012). Activation of SMO also involves a conformational switch and localization in the primary cilia of vertebrate cells. In primary cilia, a microtubule-based non motile cilium found on most vertebrate cells, SMO interacts with beta-arrestin and Kif3A in the distal tip of the cilia (Huangfu and Anderson 2005, Kovacs et al. 2008, Nozawa et al. 2013). Finally, SMO activation results in regulation of the activity of the Hedgehog signaling specific transcription factors GLI. In humans, there are three zinc finger proteins GLI. Although the three GLI proteins have similar DNA binding domains, GLI2 and GLI3 can act as transcription repressors due to their N terminal repressor domain (Aberger and Ruiz I Altaba 2014, Ruiz i Altaba 2011, Stecca and Ruiz I Altaba 2010). Indeed, GLI proteins can have different roles in the regulation of HH pathway. While GLI1 act as a transcriptional activator and thus serves as a readout of HH activity, GLI2 can act as activator or repressor and GLI3 can be a weakly activator but mainly a transcription repressor. Thus, it is the sum of the activating and repressor forms of GLI, known as the GLI code that is determinant for cell response (Aberger and Ruiz I Altaba 2014, Ruiz i Altaba 2011, Stecca and Ruiz I Altaba 2010). GLI protein activity and stability are regulated by posttranscriptional modifications. GLI1 can be for instance phosphorylated by PKA, GSK3, and CK1, enhancing its recognition by SCF family of E3 ubiquitin ligases and inducing in this manner proteasome-mediated GLI1 degradation (Jiang 2006, Riobó et al. 2006, Shi et al. 2014). PKA, GSK3, and CK1 also regulate GLI2 and GLI3 proteolysis that results in total degradation of GLI2 but only partial degradation of GLI3 and the production of a transcriptional repressor form of GLI3. GLI activators bind to the consensus sequence GACCACCCA on the DNA to induce the expression of genes related with cell cycle such as CCND1, CCND2, apoptosis like BCL2, CFLAR, transcription factors such as MYCN, FOXF1, FOXL1, proteins involved in other signaling pathways such as JAG2, GREM1, and FST, and proteins involved in the same HH pathway, resulting in a positive feedback loop in the case of GLI gene and in negative feedback loops in the case of PTCH1, PTCH2, and HHIP (Katoh and Katoh 2009).

In the brain, HH signaling is an important morphogen signaling for CNS formation, determining the differentiation of distinct brain areas and cells. The importance of SHH signaling in brain formation is revealed by the fact that the absence of this pathway in SHH knock-out mice produce the lack of ventral structures in the CNS and mice die after birth (Álvarez-Buylla and Ihrie 2014). At early embryonic stages, SHH is first expressed ventrally in the brain, in the notochord, in the precordal plate and regulates ventral hindbrain, midbrain and forebrain development (Ruiz i Altaba et al. 2002). In the ventral brain SHH pathway can induce neuron formation, controls the size of the ventral midbrain and the development of the basal ganglia. SHH is an important factor for cell growth in the brain, being

involved in oligodendrocyte formation, in regulating the size of the dorsal brain and in the cortical plate it might affect precursor cells (Ruiz i Altaba et al. 2002). Besides the role of SHH in determining the differentiation and formation of different brain regions, HH pathway has a crucial function in astrocyte formation, main glial cells in the brain. HH pathway is a key signaling for astrocyte formation by promoting progenitor differentiation into astrocytes. Indeed, progenitor cells expressing SHH contribute to both, neurons and astrocytes production in a caudal area of the brain. However, SHH expressing progenitors suffer a gradual shift from neurogenesis to gliogenesis, generating mainly hypothalamic astrocytes in later development phases (Alvarez-Bolado et al. 2012). Additionally, HH signaling is involved in astrocyte generation from other progenitor cells in the brain. In the case of radial astrocytes, HH pathway inhibition, by the absence of primary cilia, an organelle essential for HH signaling in mammals, or the absence of SMO, prevents radial astrocytes development (Han et al. 2008). In progenitors cells isolated from the dorsal telencephalon, and the developing optic nerve, SHH favors astrocyte generation and proliferation (Araújo et al. 2014, Wallace and Raff 1999). In the adult brain, germinal niches that include the ventricular subventricular zone **(V-SVZ)** and the subgranular zone **(SGZ),** continue to produce neurons and glial cells. In the V-SVZ, astrocyte-like neural stem cells express GLI and thus respond to SHH secreted in this environment (Ihrie et al. 2011, Palma et al. 2005). Finally, HH signaling plays a role in the differentiation and maturation of astrocytes such as adult cerebellar Bergmann glia astrocytes and mouse cerebellar granule cell precursors form the proliferative zone of the external germinal layer (Marazziti et al. 2013, Okano-Uchida et al. 2004). Once differentiated, SHH signaling, regulated by the combination of GLI transcriptions factors in their activator and inactivator forms, is important for proper astrocyte functions like the release from neurotransmitters and for maintaining the blood brain barrier (Alvarez et al. 2011, Okuda et al. 2015, Petrova et al. 2013). Furthermore, SHH signaling regulates glutamate and ATP release from astrocytes, and thus is essential for astrocyte metabolism and metabolic support to neurons (Okuda et al. 2015). SHH signaling is then an important pathway not only for astrocyte generation in the brain but also for astrocyte differentiation and function. If HH signaling is crucial for brain formation and astrocyte generation during development, and in physiological conditions, what is the implication of this signaling upon brain injury?

Brain repair requires astrocyte activation through SHH signaling. Besides giving ionic and metabolic support to neurons, regulating synapse neurotransmission, and regulating blood brain barrier, astrocytes play key roles upon brain injury. Initially observed in multiple sclerosis specimens, astrocytes that react to CNS changes present a different appearance, and were denominated "reactive astrocytes". Reactive astrocytes have both, biochemical and morphological changes, upon brain injury and in pathological conditions. In addition to hypertrophy and increased expression of Glial Fibrillary Acidic Protein **(GFAP),** reactive astrocytes secrete more cytokines, growth factors and extracellular matrix components (Robel and Sontheimer 2015). Upon brain injury conditions such as those found in pathological situations, some astrocytes can proliferate and acquire an immature phenotype, that may be related with a progenitor state that can reestablish damaged cells in the brain (Robel and Sontheimer 2015). Although evidences *in vivo* of the potential of astrocytes as progenitor cells in the adult brain remain elusive (Dimou and Götz 2014), the reaction of astrocytes known as astrogliosis, is one of the most important reactions in the brain upon injury and is found in diverse situations like mesiotemporal lobe epilepsy and Alzheimer´s disease (Chung et al. 2015, Robel and Sontheimer 2015). Albeit cell migration was thought to be one of the characteristics of reactive astrocytes to reach injury sites, recent investigations *in vivo* have shown that astrocytes do not migrate towards injury site. Indeed, astrocyte response in the brain is heterogeneous, with astrocytes that do not change cell morphology, astrocytes that direct their process toward the lesion site and astrocytes that proliferate (Bardehle et al. 2013, Zamanian et al. 2012). Thus, instead of migration upon acute injury, astrocytes can extend their cytoplasm towards the wound site or proliferate in close proximity to the vascular system of the brain (Bardehle et al. 2013).

Interestingly, in many tissues, injury repair brings out biological processes that recapitulate tissue development and that enable tissue re-formation. For instance, cell signaling pathways operative during brain development like Sonic Hedgehog pathway, are reactivated upon brain injury as will be outlined below. Given the importance of Sonic Hedgehog pathway for brain development, it is very interesting to point out evidences *in vitro* and *in vivo* of the re-activation of this pathway in the adult brain upon tissue injury (annex1). In many cases, astrocyte activation is due to mechanical, chemical or biological injury and this astrogliosis is in part, mediated by SHH signaling (figure 1).

Biological agents like *Angiostrongylus cantonensis,* an important etiologic agent of eosinophilicmeningitis or

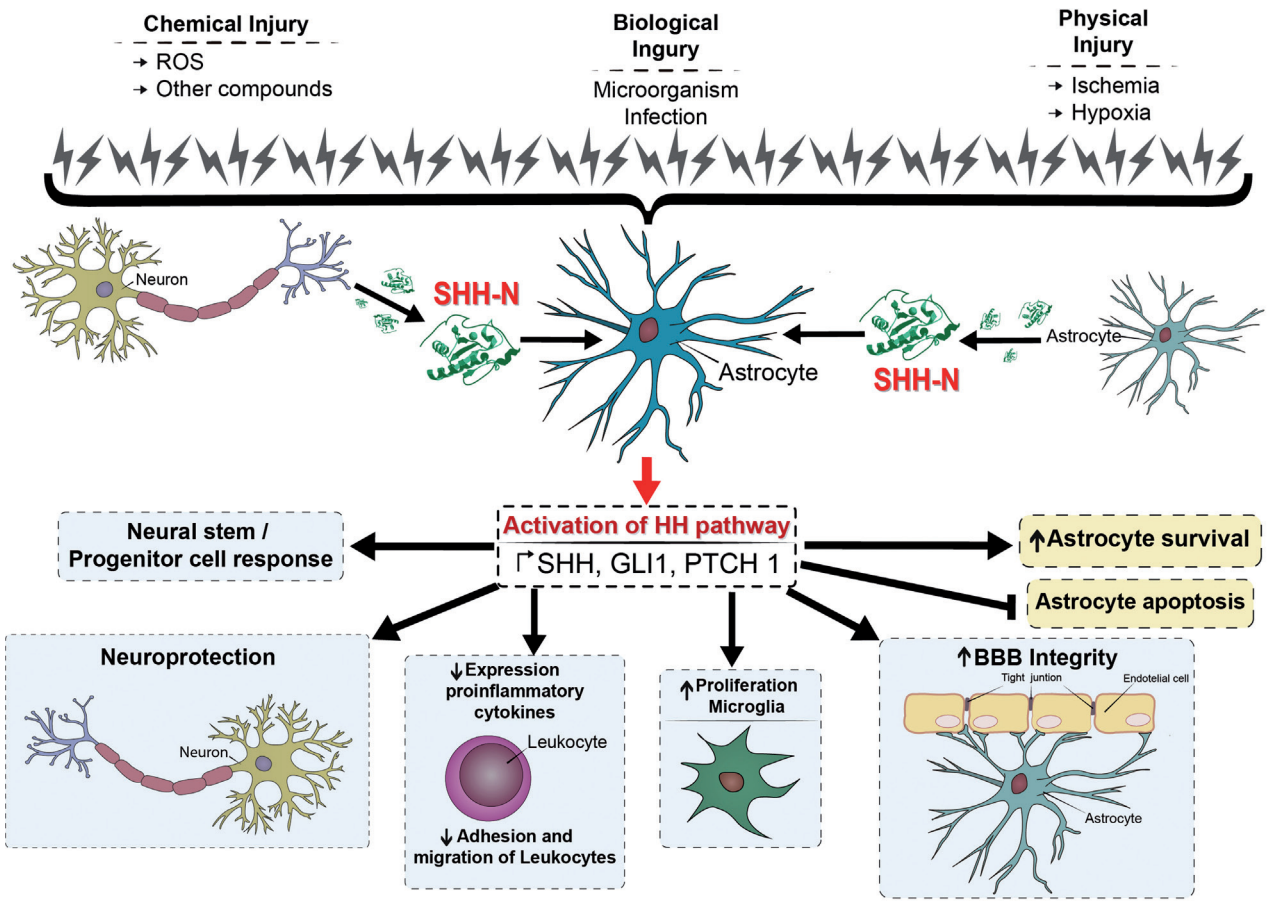

Figure 1. Brain injury activates Sonic Hedgehog **(SHH)** signaling pathway. Chemical, biological and physical injuries elicit a communication between neurons and astrocytes through SHH. Astrocyte response, mediated by SHH signaling, mediates different cell responses on neurons, leukocytes, microglia, endothelial cells and astrocytes that may represent the initial steps of tissue regeneration in the neurovascular unit and in the brain parenchyma

eosinophilic meningoencephalitis in humans, have been found to induce SHH signaling. Cocultures of astrocytes with living fifth-stage larvae or soluble antigens, increased GFAP and SHH expression. Importantly, SHH enhanced astrocyte survival, probably by reducing BCL2-dependent cell death (Chen et al. 2015). Besides brain injury provoked by biological agents, mechanical injury can also elicit a SHH signaling that may be related with tissue repair. *In vitro*, scratches of monolayer astrocyte cultures produce an increase in SHH production by astrocytes, the loss of astrocyte markers such as GFAP and S100, and the expression of Neural stem cells proteins like nestin, Sox2, and CD133. Furthermore, supernatant of injured astrocytes containing SHH up regulates not only SHH but also PTCH, Gli2, and cyclin D expression in astrocytes, putting in evidence the complete activation of the SHH pathway in these cells (Yang et al. 2012) (figure 2). Experiments *in vivo* have also proved the activation of SHH signaling in different settings of brain injury and its implication in tissue repair. Different types of injury appear to elicit different HH signaling and astrocyte responses *in vivo*. Comparing ischemic lesion, traumatic injury, progressive (chronic) amyloid plaque deposition, and a noninvasive model of widespread neuronal death, Sirko et al. found that only invasive injury, such as stab wounding or cerebral ischemia induce a de-differentiation process of astrocytes. In this process, astrocytes acquire neural stem cells characters, by SHH signaling cascade, necessary and sufficient for this response *in vitro* and *in vivo* (Sirko et al. 2013). If SHH mediates astrocyte reactivity in invasive injury situations in the brain, induction of this pathway can be associated with tissue repair. In two different

spinal cord injury models *in vivo*, by contusion and dorsal hemioversection, sustained controlled delivery of SHH in injury areas enhances proliferation of NG2+ cells and decreases astrocytic scar formation (Lowry et al. 2012) (figure 2). In the brain, one of the critical parameters for cell maintenance is the permanent supply of oxygen. In the absence of appropriate oxygen concentrations, cells can rapidly undergo cell death. Under restriction in blood supplies or ischemia, irreversible brain damage associated with cerebral hypoxia and glucose deprivation can lead to stroke as fast as 5 minutes later at human body temperature. Interestingly, cerebral hypoxia induces SHH expression on neural progenitor cells and neurons that promote cell proliferation (Sims et al. 2009). Additionally, astrocytes respond to oxygen-glucose deprivation by secreting SHH that promotes the proliferation, migration of microvascular endothelial cells and tube formation in coculture models, in a RhoA and ROCK-dependent manner (He et al. 2013). In mice experiments, cortical ischemia upregulates SHH expression in neurons, in reactive astrocytes and in nestin-expressing cells in the cortical area near the injury site and the adjacent striatum (Jin et al. 2015). In these conditions, SHH signaling promotes tissue stability and injury repair. Furthermore, after stroke, SHH treatment reduces behavioral impact on animals, enhancing multiple horizontal movement parameters compared to vehicle treated mice (Jin et al. 2015). SHH signaling also decreases brain edema and preserves blood-brain barrier **(BBB)** permeability (Xia et al. 2012), essential for brain function. BBB is formed by capillary endothelial cells, pericytes, and perivascular astrocytes that create a highly selective permeability barrier protecting the neural tissue from variations in blood composition and toxins. SHH produced by astrocytes plays an important role in maintaining BBB integrity, by upregulating the expression of tight junction proteins. Upon inflammatory conditions, IL1 beta reduces BBB integrity by suppressing astrocyte SHH release (Wang et al. 2014). In animal ischemia models, increased SHH secretion increases Ang-1 expression in astrocytes and correlates with increased ZO-1 and occluding expression in primary brain microvessel endothelial cells, enhancing tight junction stability and avoiding BBB disruption (Xia et al. 2012) (figure 2). Importantly, endothelial brain cells express HH receptor PTCH, and HH pathway has been found to decrease the expression of proinflammatory mediators and to decrease the adhesion and migration of leukocytes, promoting the immune quiescence of BBB endothelial cells, providing a barrier effect (Alvarez et al. 2011). If the absence or the decrease of oxygen concentration represent a critical situation for neuron and astrocyte survival, the presence of reactive oxygen species can also elicits a stress response mediated by SHH signaling. Upon oxidative stress SHH pathway is (re)-activated as proven by increased levels of SHH, PTCH1, and GLI1 in astrocytes treated with 100 mm of H_2O_2 for 24 hours. HH signaling activation on astrocytes enhances AKT phosphorylation, has a pro-survival effect and a protective effect on cocultured neurons (Xia et al. 2012). Excess of other molecules in the brain, such as kainic acid, can represent another source of brain injury. Kainic acid is an analog of the excitatory amino acid L-glutamate and can induce neuron death in the central nervous system. In a model of kainic acid neurodegeneration, SHH expression is upregulated in astrocytes, along with increased Gli activity and astrocyte proliferation, independently of the severity of neurodegeneration (Pitter et al. 2014)

Upon different types of brain injury, Hedgehog signaling and astrocyte activation appear thus to be essential for brain response (annex 1). After injury, Hedgehog signal may be secreted by neurons and received by glial cells such as astrocytes (figure 1). *In situ* hybridization studies of adult mouse cerebellum have revealed that while SHH is expressed in HuC/D-positive neurons, HH-receptor Ptch1 is expressed in S100β-positive astrocytes, suggesting that SHH mediates paracrine signaling between neurons and astrocytes. Other studies in mice have shown that SHH is also produced by Purkinje neurons (Fleming et al. 2013). Thus, in the first steps of cell reaction to brain injury, SHH pathway may represent a paracrine signaling between neurons and astrocytes that elicits tissue repair. Once activated, astrocyte may communicate through SHH signaling to other glial cells, including astrocytes, to orchestrate a coordinated cell response upon injury (figure 2).

CONCLUSIONS

SHH signaling is an essential pathway for brain patterning and cell differentiation during development. However, studies in adult organisms have highlighted the importance of this signaling pathway in the interplay between neurons and glial cells. Among glial cells, astrocytes play a key role for the regulation of metabolism, neurotransmitter clearance, blood brain barrier and synapse maturation, plasticity and elimination, and thus for brain function. Upon injury, astrocytes exhibit specific cell responses that include cell proliferation and activation of stem cell features mediated in part by SHH signaling. Once activated by SHH, astrocytes coordinate tissue repair, regulating astrocyte and neuron survival, the integrity of blood brain barrier and

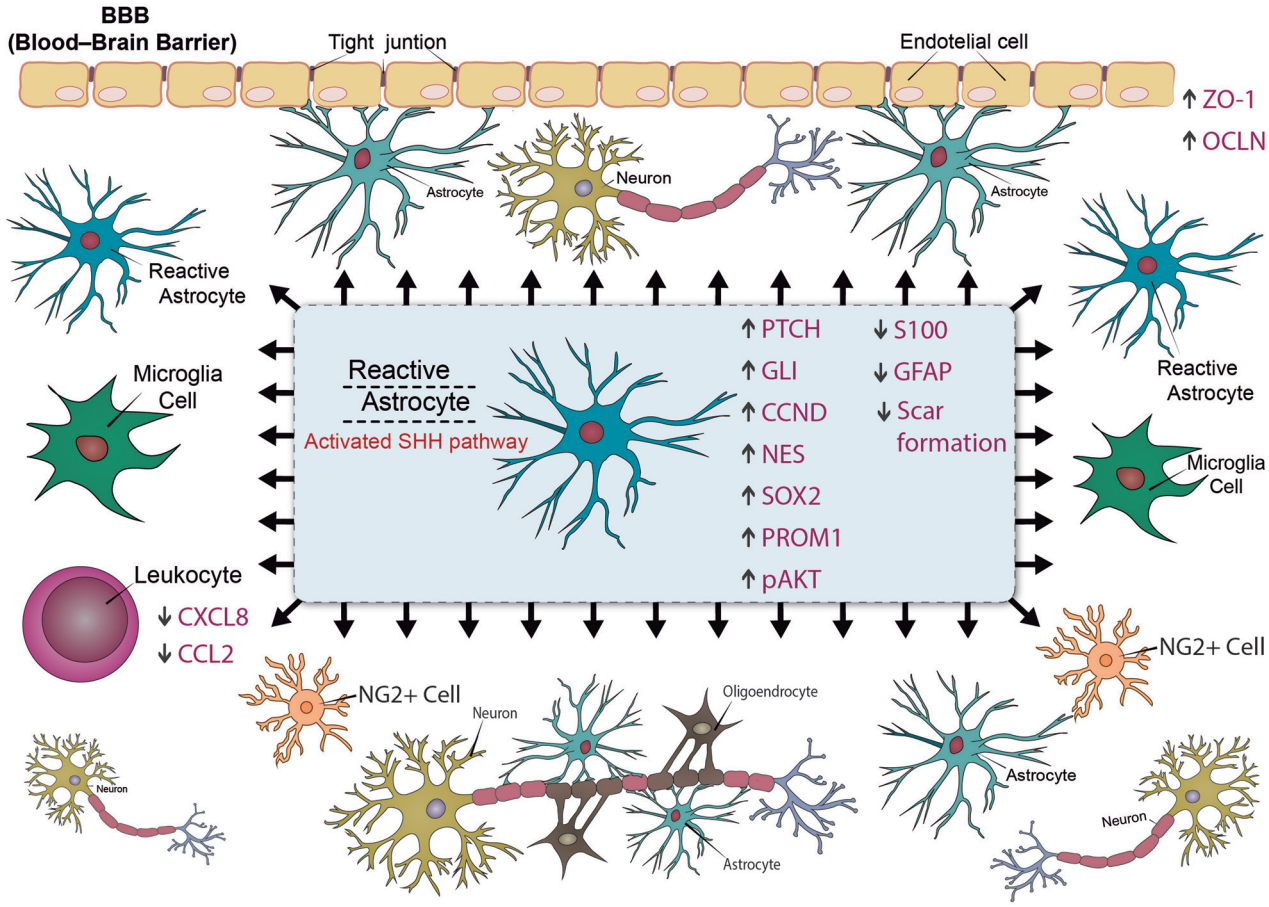

Figure 2. Astrocyte response to brain injury is mediated by Sonic Hedgehog **(SHH)** signaling pathway, enabling a coordinated cell reaction in the tissue. Upon brain injury, astrocytes become reactive and activate SHH pathway, resulting in the upregulation of genes related with SHH signaling pathway (PTCH, GLI), with cell cycle (CCND), with cytoskeleton (NES), with progenitor state (SOX2, PROM1) and in increased levels of AKT. Furthermore, SHH activation in astrocytes correlates with a decreased expression of S100 and GFAP and a reduction in scar formation. SHH signaling activation upon brain injury enhances proliferation of microglia, oligodendrocytes and NG2 positive cells, neuron survival and blood brain barrier integrity through the upregulation of ZO-1 and OCLN expression in microvessel endothelial cells, orchestrating in this manner a coordinated tissue response for brain repair

microglia activity. Thus, by regulating astrocyte activity, SHH pathway appears as a key player, *in vitro* and *in vivo*, for tissue repair. If this link is supported by reliant experimental evidence, many questions remain to be solved. On one hand, novel findings should bring to light the importance of SHH in the dynamic communication between neurons and astrocytes, and other glia cells, for efficient tissue response upon injury. On the other hand, it will be necessary to determine if SHH pathway is related with brain repair in acute injury and if upon chronic brain injury, long-term activation of this pathway may contribute to brain diseases. Finally, it will be of great interest to understand if SHH signaling elicits the activation of other signaling modules in astrocytes and neurons that may compose the signaling reactions that enables brain cell reaction, and if modulating this signaling network may enhance brain repair in the context of brain injury in different neurological diseases including cancer.

ACKNOWLEDGMENTS

I am very grateful to Andrés Mauricio Díaz Vallejo for his collaboration in figures design. Olga María Bermúdez Muñoz is currently funded by CODI (Comité para el Desarrollo de la Investigación) of the Universidad de Antioquia (Fondo de Apoyo al Primer Proyecto, project # 668).

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Annex 1. Experimental models of Sonic Hedgehog **(SHH)** signaling in astrocytes upon brain injury

Type of injury	Experiment model	Results	Reference
Biological (infection)	Co-culture of astrocytes with living fifth-stage larvae or soluble antigens of *Angiostrongylus cantonensis*.	- Shh expression is increased in astrocyte cocultured with living fifth-stage larvae or soluble antigens. - Astrocytes pretreated with SHH have increased survival in presence of larva antigens and the opposite effect is observed with the SMO inhibitor cyclopamine. - Astrocytes pretreated with SHH have increased expression of BCL2 and GRP78 that may inhibit cell death in presence of *A. cantonensis* in the brain.	Chen et al. 2015
Physical	Scratches of monolayer astrocyte cultures.	- Increase of SHH production in astrocytes. - Astrocytes lose their immunophenotypical profile and gain NSPC (neural stem/progenitor cells) characteristics when astrocytes are incubated with the injured astrocyte conditioned medium (ACM). In these conditions, astrocytes exhibit increased expression of SHH, PTCH, GLI2 and Cyclin D.	Yang et al. 2012
Physical	Spinal cord injury in mice by contusion and dorsal hemioversection.	- Sustained delivery of SHH increases proliferation of endogenous NG2+ cells and of oligodendrocyte cells, decreases astrocyte scar formation, and increases corticospinal and raphespinal tract fibers, after spinal cord injury.	Lowry et al. 2012
Physical	Two models of invasive brain injury: stab wounding and cerebral ischemia. Two models of non-invasive brain injury: chronic amyloidosis and neuronal death in mice.	- Only invasive models induce reactivity in astrocytes, eliciting stem cell response *in vitro* and *in vivo*, through SHH signaling.	Sirko et al. 2013
Physical	Distal MCA (Middle cerebral artery occlusion) model in mice.	- Upon brain injury, SHH expression is increased in neurons, reactive astrocytes and nestin-expressing cells of the cortical area, near the injury site and the adjacent striatum. In the subventricular zone (SVZ), SHH pathway genes are also expressed. - While conditional deletion of SHH in nestin-expressing cells correlates with more severe behavioral deficits in mice upon brain injury, SHH agonist SAG reduces behavioral deficits in mice after brain injury.	Jin et al. 2015

Continuation: Annex 1

Type of injury	Experiment model	Results	Reference
Physical and hypoxia	- Brain ischemia model: Male SV129 mice exposed to a 20-minute middle cerebral artery occlusion. - Hypoxia model: Primary cell cultures of neurons, astrocytes, and NPCs (neural progenitor cells) exposed to 16 hours of hypoxia (1% O_2).	- Middle cerebral artery occlusion results in SHH and GLI1 mRNA increased expression and increase in SHH protein levels in the hippocampus. - The proliferation of NPC is increased under hypoxic conditions *in vitro*, and is enhanced by the presence of recombinant SHH and blocked by SMO inhibitor cyclopamine.	Sims et al. 2009
Hypoxia	Coculture of brain microvascular endothelial cells (BMECs) with astrocytes, under oxygen deprivation condition (1% O2).	- SHH secretion by astrocytes is increased under oxygen deprivation. - SMO inhibitor cyclopamine and 5E1 SHH antibody decrease proliferation, migration and tube formation of BMECs cocultured with astrocytes after oxygen deprivation.	He et al. 2013
Chemical (Reactive oxygen species)	Primary culture of rat astrocytes treated with H_2O_2. Coculture of astrocytes and neurons, treated with H_2O_2.	- H_2O_2 treatment in primary cultured astrocytes increases the mRNA and protein levels of SHH, PTCH1, and GLI1 mainly in astrocytes but also in neurons. - H_2O_2 treatment induces astrocyte cell death, that is enhanced by SMO inhibitor cyclopamine and by SHH anbitody 5E1, and decreased by exogenous SHH. - In the coculture model of astrocytes with neurons, the SHH antibody 5E1 blocks the neuroprotective activity of astrocytes on neurons.	Xia et al. 2012
Chemical	Astrocytes treated with IL-1 β.	- IL-1β decreases SHH production in astrocytes, affecting their role in BBB integrity - While astrocyte conditioned media, SHH, or SHH signal agonist upregulates the expression of tight junction proteins and thus BBB integrity, SMO inhibitor has the opposite effect.	Wang et al. 2014
Chemical	Kainic acid- (KA) induced neurodegeneration by intraperitoneally injection in mice.	- KA produces an increased expression of SHH and GLI activity in astrocytes. - Astrocyte secreted SHH increases the activation and proliferation of astrocytes and microglia in the injured brain.	Pitter et al. 2014
Gene silencing	Pharmacological inhibition and genetic inactivation of the HH signaling pathway in endothelial cells.	- HH pathway decreases the expression of proinflammatory mediators and the adhesion and migration of leukocytes, *in vivo* and *in vitro* in endothelial cells of the blood brain barrier.	Alvarez et al. 2011

Actual Biol 38 (105): 197-209, 2016 | DOI: 10.17533/udea.acbi.v38n105a07