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Review Article

Notch signaling and neurogenesis in normal and stroke brain

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Abstract: Adult neuronal stem cells (NSCs) hold great promise for brain repair because of their unique location within the central nervous system, their potential to proliferate and to differentiate into all major neural lineages, and their ability to functionally incorporate into existing neuronal circuitry after stroke. Nevertheless, the ability to exploit these cells for therapeutic purposes is hampered by the lack of knowledge about the signals that control the generation of a functional neuron from adult NSCs after stroke, particularly in the aged brain. Therefore, to further define the regulatory mechanisms that underlie neurogenesis after stroke, it is critically important to develop future NSC-based repair strategies. Notch signaling defines a fundamental pathway controlling cell fate acquisition. Studies have shown that Notch signaling pathways play critical roles during the maintenance, proliferation, and differentiation of NSCs in the developing brain. Recent evidence shows that Notch1 signaling is conserved in the regulation of adult neurogenesis. Here we summarize current knowledge about the role of Notch signaling in the regulation of neurogenesis in the normal and stroke brain.

Key words: Neurogenesis, adult, Notch signaling, SVZ, stroke

Introduction

Stem cells are characterized by self-renewal and multi-lineage differentiation. Neural stem cells (NSCs) in the adult brain hold great promise for brain repair because of their unique location within the central nervous system (CNS), potential to proliferate and to differentiate into all major neural lineages, and ability to functionally incorporate into the existing neuronal circuitry. However, the capacity of such self-repair is limited. The successful development of NSC recruitment therapy will depend on our ability to manage the proliferation, migration, differentiation and functional integration of recruited cells. The first step towards this goal is to investigate the molecular mechanisms governing NSC behaviors. Therefore, to further define the regulatory mechanisms that underlie neurogenesis in the adult brain, it is critically important to develop future NSC-based repair strategies for treating neurological diseases such as stroke [1].

Growing evidence shows that biological behaviors, such as proliferation, migration and differentiation, of NSCs in the adult brain must be properly regulated by intrinsic signals and external factors [2-4]. Notch signaling defines a fundamental pathway controlling cell fate acquisition [5]. For example, Notch signaling pathways play critical roles during the maintenance, proliferation, and differentiation of NSCs in the developing brain. Recent studies show that Notch1 signaling is conserved in the regulation of adult neurogenesis. Here, we summarize the current knowledge about the role of Notch signaling in the regulation of neurogenesis in the adult brain under normal and pathological conditions such as stroke.

Notch family

The *Notch* gene was discovered in 1917 by Thomas Hunt Morgan, when it was first noticed in a strain of the fruit fly *Drosophila melanogaster* with notches apparent in their wing blades. However, its sequence was not determined until the 1980s. Studies show that the Notch signaling family is composed of a group of highly conserved proteins. So far, a number of Notch signaling family members, including Notch receptors, ligands and their corresponding intracellular signaling molecules have been identified.

Notch receptors in mammals

In mammals, the *Notch* genes encode large transmembrane proteins that act as receptors for the DSL (Delta, Serrate, Lag-2) family of ligands. There are four Notch membrane-bound type I receptors, referred to as Notch1, Notch2, Notch3, and Notch4 (**Figure 1A**). The Notch proteins are expressed on the cell surface as heterodimers composed of an extracellular region, a single transmembrane-pass, and a small intracellular region [5, 6]. The extracellular domain contains a variable number of epidermal growth factor (EGF)-like repeats, which are followed by three cysteine-rich LIN12/Notch repeats (LNR) that prevent signaling in the absence of the ligand [7, 8]. The Notch intracellular domain (NICD) contains a RAM23 domain [9], seven Ankyrin/CDC10 repeats involved in protein-protein interactions [10], and a PEST sequence [rich in proline (P), glutamic acid (E), serine (S) and threonine (T)] that negatively regulates protein stability [11]. In addition, Notch receptors 1-3 contain two nuclear localization signals (NLS) compared to one NLS in Notch4. The NSL is necessary to target the intracellular domain to the nucleus where the transcriptional activation domain (TAD) activates downstream events. Note that Notch3 and Notch4 contain no TAD domain.

Notch ligands in mammals

In flies, the two structurally related Notch ligands, Delta and Serrate, have both redundant and nonredundant functions [5, 12]. Notch ligands are conserved in vertebrates and include the Serrate orthologs Jagged1 and 2 and the Delta orthologs Delta1, 3, and 4. In mammals, five structurally similar Notch ligands (Delta-like1, Delta-like3, Delta-like4, Jagged1, and Jagged2) have been identified (**Figure 1B**). Delta-like1, 3 and 4 are homologs of *Drosophila* Delta (dDelta), while Jagged1 and 2 are homologous to *Drosophila* Serrate (dSerrate). All Notch ligands are single pass transmembrane polypeptides, and their intracellular domains vary in length and do not display any significant sequence similarity except at the very end of the C-terminal. Notch ligands consist of multiple highly conserved EGF-like motifs and a conserved DSL domain [13]. In addition, Jagged1 and Jagged2 harbor an additional cysteine-rich domain. Because most ligands are also transmembrane proteins, the extracellular domain of Notch ligands interacts with Notch receptors expressed on neighboring cells that are in direct contact [13]. This way, groups of cells can organize themselves, such that, if one cell expresses a given trait, this may be switched off in neighboring cells by the inter-cellular Notch signal. Notably, there is little proof that Delta-like3 physically binds to the Notch receptors or that it truly functions as a Notch ligand [14].

Notch signaling

The Notch pathway is a complex signaling system, composed of a series of molecular events (**Figure 2**). Experiments in many different systems have provided a detailed model for Notch signaling that involves ligand-dependent cleavages of both the extracellular and intracellular domains of the receptor [15]. Notch signaling is initiated by direct cell-cell interactions that facilitate binding between the transmembrane Notch ligand Delta or Jagged on a signaling cell and the Notch receptor on a responding cell, leading to consecutive proteolytic cleavages of the receptor. The first cleavage occurs in the Golgi by the furin convertase enzyme (S1 cleavage), which results in the expression of a non-covalently linked Notch heterodimer receptor on the cell surface [16, 17]. In the absence of Notch ligand interaction, the cytoplasmic adaptor protein Numb interacts directly with the cytoplasmic domain of Notch and inhibits Notch activation. On the cell surface, proteolytic cleavage (S2 cleavage) by the TNF-α-Converting Enzyme (TACE) and ADAM17 metalloprotease occurs extracellularly (residue 1711). After cleavage, the extracellular portion of Notch continues to interact with the ligand and is then endocytosed by the ligand-expressing cell. After this first cleavage, γ-secretase, a complex composed of four

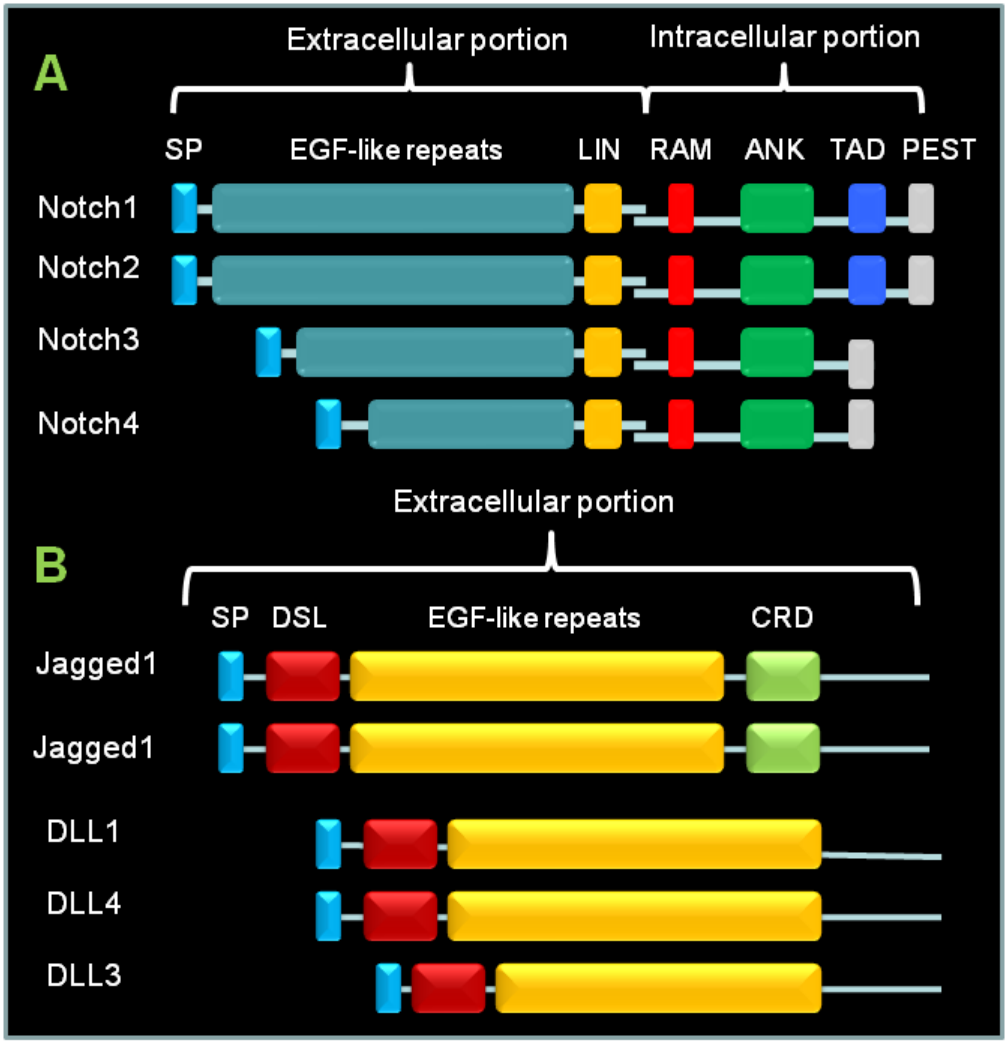

Figure 1. Schematic representation of the Notch receptor and ligand structure in mammals. **A)** There are four mammalian Notch receptors (Notch1–Notch4). Mature Notch proteins are comprised of the extracellular and transmembrane (intracellular) portions. Extracellular Notch proteins are characterized by numerous EGF-like repeats. Transmembrane Notch1 and Notch2 are almost identical in size and share many structural features, including the membrane-proximal RBP-J-associated molecule (RAM) domain, the Ankyrin (ANK) domain, two nuclear-localization sequences (NLSs); a carboxy-terminal transactivation domain (TAD), and a PEST (proline-, glutamate-, serine- and threonine-rich) domain. Transmembrane Notch3 and Notch4 are shorter and lack the TAD. The heterodimerization domain (HD) spans the region of interaction between the extracellular and transmembrane portions. **B)** Mammals have five mammalian Notch ligands (Jagged1, Jagged2, Delta-like 1 (DLL1), DLL3 and DLL4). Delta-like1, 3 and 4 are homologs of *Drosophila* Delta (dDelta), while Jagged1 and 2 are homologous to *Drosophila* Serrate. Notch ligands are transmembrane proteins of which the extracellular domain contains a characteristic number of EGF-like repeats and a cysteine rich N-terminal DSL domain. Jagged1 and Jagged2 contain an additional cysteine rich domain (CRD). SP: signal peptide.

different integral membrane proteins (presenilin, nicastrin (Nct), Aph-1, and Pen-2) cleaves the remaining part of the Notch protein just inside the inner leaflet of the cell membrane of the Notch-expressing cell (S3 cleavage). This occurs within the transmembrane domain (residue 1744) and leads to the release of the NICD into the cytoplasm [18, 19]. NICD subsequently translocates to the nucleus, where it binds via its RAM23 domain to the transcription factor CSL (CBF1 in humans, Suppressor of Hairless

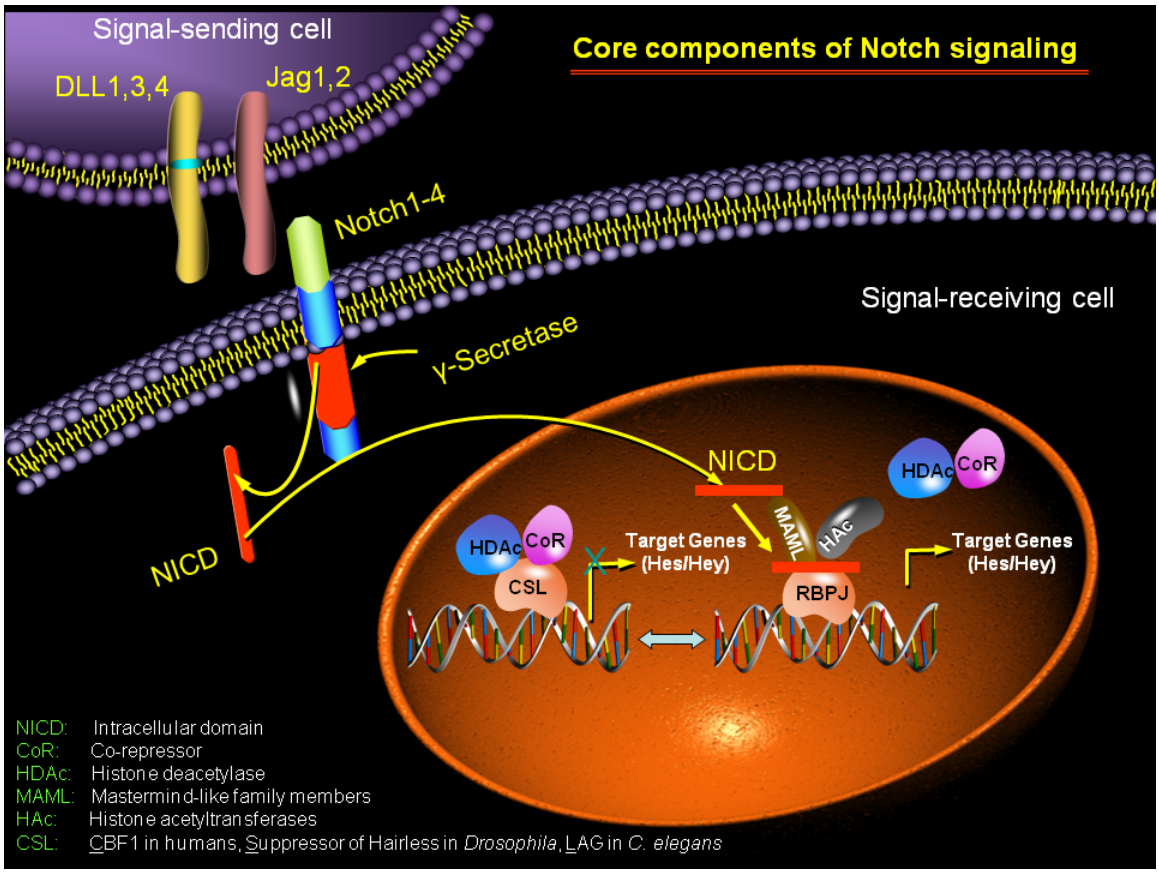

Figure 2. Schematic representation of the Notch signaling in mammals. Notch signaling is triggered upon ligand-receptor interaction which induces two sequential proteolytic cleavages, the first in the extracellular domain mediated by metalloproteases of the ADAM family, and the second within the transmembrane domain mediated by a γ-secretase activity of presenilins (PS). This second cleavage allows the release of the NICD, which translocates to the nucleus and associates with the CSL family transcription factor complex, resulting in subsequent activation of the notch target genes Hey and Hes family members.

in *Drosophila*, LAG in *C. elegans*), also called RBPJK (Recombination Signal-Binding Protein 1 for J-Kappa) in mice [20]. CSL is bifunctional. In the absence of NICD, CSL binds to at least four co-repressors, the silencing mediator of retinoid and thyroid hormone receptor (SMRT), histone deacetylase-1 (HDAC1), KyoT2, and Ski-interacting protein (SKIP), which suppresses transcription [21]. In contrast, the interaction of CSL with the RAM23 and ANK repeats of the NICD displace these repressors to generate a transcriptional activator complex, which in turn regulates expression of Notch target genes [5]. The nuclear protein Mastermind-like (MAML) also interacts with this complex to further increase transcription. However, the truncated version of MAML that maintains an association with the complex, behaves in a dominant-negative (DN-MAML) fashion and inhibits Notch activation. The most widely accepted Notch/CSL targets are members of the basic helix-loop-helix (bHLH) hairy/enhancer of the split (Hes) family and the related HRT/Herp (Hes-related repressor protein) transcription factor family [22-24]. Other proteins also participate in the intracellular portion of the Notch signaling cascade.

Notch signaling and cell fate

Notch signaling regulates a wide variety of mammalian cell fates and processes, and plays a fundamental role in development. Signals exchanged between neighboring cells

through the Notch receptor can amplify and consolidate molecular changes that eventually dictate cell fates. Thus, Notch signals control how cells respond to intrinsic or extrinsic developmental cues that are necessary to unfold specific developmental programs. Notch signaling also has a role in the following processes: 1) stabilization of arterial endothelial fate and angiogenesis. The finding that Notch genes are robustly expressed in the vasculature suggests roles for Notch in guiding endothelial and associated mural cells through the myriad of cell-fate decisions needed to form the vasculature [25, 26]. In fact, mice with defects in genes encoding Notch, Notch ligands, and components of the Notch signaling cascade invariably display vascular defects. For example, the survival of Notch4-deficient mice shows that Notch4 is dispensable for vascular development [27], while expression of an activated form of Notch4 within the endothelium disrupts normal vascular development [28, 29]. In addition, mutations in Notch3 or Jagged1 lead to human cardiovascular diseases: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and Alagille syndrome (paucity of intrahepatic bile ducts with cholestasis, cardiac disease, skeletal abnormalities, ocular abnormalities, and characteristic facies), respectively [30]. 2) Bone regeneration and osteoporosis. Notch activation reduces the surface expression of c-Fms, a receptor for macrophage colony-stimulating factor, in osteoclast precursor cells and enhances the expression of osteoprotegerin in stromal cells, which results in the down-regulation of osteoclastogenesis [31]. In addition, the hematopoietic stem cell compartment is expanded during bone development and participates in commitment to the osteoblastic lineage, suggesting a potential role for Notch in bone regeneration and osteoporosis [32]. 3) Roles in hematopoietic and immune systems. Notch signaling has a key role in the process of lymphocyte development. Notch signaling specifies T cell lineage fate, and controls several early steps of T cell development, as well as specific cell fate and differentiation decisions in other hematopoietic lineages [33]. Notch signaling is necessary for the self-renewal of hematopoietic stem cell/progenitor cell and the onset of definitive hematopoiesis in the embryo as well [34, 35]. 4) Notch signaling is a key determinant of muscle regenerative potential that declines with age [36]. It also has a role in the following processes: neuronal function and development, cardiac valve homeostasis, regulation of cell-fate decision in mammary glands. Faulty Notch signaling is implicated in many diseases including T-ALL (T-cell acute lymphoblastic leukemia), Multiple Sclerosis, Tetralogy of Fallot, Alagille syndrome and a myriad of other disease states.

Adult neurogenesis in adult brain

Although Altman first observed the proliferative potential of the adult rodent brain in the 1960s, it has been thought for some time that the brains of adult mammals do not generate new neurons [37, 38]. After several years of debate, it is now accepted that the rostral subventricular zone (SVZ) surrounding the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) are active proliferative regions that generate neurons [39], astrocytes [40] and oligodendrocytes [39, 41] continuously throughout life in mice [42], rats [43], non-human primates [44] and humans [45]. NSCs in the SVZ of the adult brain can be identified by ^{3}H-thymidine and thymidine analog BrdU that is incorporated into the DNA of S-phase cells [37, 46], by expression of neural stem/progenitor marker such as nestin [47], and by their ability to form neurospheres that give rise to multiple cell types in vitro [48]. Four cell types have been identified in adult SVZ: (1) neuroblasts, or Type A cells; (2) astrocytes, or Type B1 and B2 cells; (3) undifferentiated, or Type C cells [49]; and (4) ependymal cells [40, 41]. The SGZ, a thin lamina between the hilar region and the granule cell layer of the hippocampal DG, retains the potential to form new neurons into adulthood [50, 51].

Stem cells in the SVZ generate immature neurons that aggregate to form an extensive network of neuroblast chains along the lateral wall of the lateral cerebral ventricle [52]. These chains of neuroblasts form a highly restricted migratory route, called the rostral migratory stream (RMS), which extends from the anterior SVZ into the olfactory bulb (OB). Unlike the radial glial-guided migration of young neurons during early brain development [53], neuroblasts undergo "chain migration" in the adult SVZ/RMS and migrate along one another, which involves interactions between

the migrating cells and tube-like structures formed by specialized astrocytes [54]. Most of the neurons born in the adult SVZ migrate over a great distance to the OB through the RMS (2-6 Days). After immature neurons reach the OB, they begin to differentiate into two different types of local interneurons (15-30 Days). Over 95% differentiate into GABAnergic granule neurons whereas the remainder become periglomerular neurons expressing either GABA and/or dopamine as a neurotransmitter. Newborn granule cells and periglomerluar neurons become integrated into the OB circuitry and respond to olfactory stimuli (15-30 Days). Newborn granule cells can be classified into cells with dendrites that do not extend beyond the mitral cell layer and cells that possess non-spiny dendrites reaching into the external plexiform layer, whereas other cells in the SVZ may die shortly after their genesis [55-57]. However, the OB is not essential for proliferation and directed migration of SVZ precursors, since the proportion of dividing or dying cells in the RMS was not significantly affected after olfactory bulbectomy [58].

NSCs in the SGZ migrate into the granular cell layer (GCL) and undergo neuronal differentiation. However, the speed of maturation varies between neurons. The newborn cells eventually become physiologically indistinguishable from fully mature neurons (over 2 months old). In rodents, the rate of neurogenesis declines with age [51, 59, 60], but the number of granule cells in the DG increases into midlife and reaches a plateau thereafter [61]. Neurogenesis persists in the DG in elderly rodents [62] and humans [63] to maintain an equilibrium between the production of newborn cells and neuronal loss. Hippocampal neurogenesis in aged mice living in an enriched environment is higher by fivefold than in controls with significant improvements of learning parameters, exploratory behavior, and locomotor activity, suggesting that the old brain still has the ability to acutely react to functional challenges with a neurogenic response [64].

Role of Notch signaling in neurogenesis in normal brain

Notch receptors, in combination with other cellular factors, affects the implementation of differentiation, proliferation, and apoptotic programs at all stages of development [5]. Notch signaling inhibits neuronal differentiation in vertebrates and invertebrates [5, 65] as well as suppresses oligodendrocyte development from precursors during gliogenesis [66]. In the vertebrate CNS, Notch receptors and their ligands are expressed in the proliferative zones of undifferentiated cells [67] and multiple *Notch* genes are expressed in all or most NSCs of the developing zebra fish neural tube [68]. Jagged homologues have been identified as ligands of Notch receptors [69]. The concurrent knockdown of multiple Jagged homologs results in a phenotype that serves as a model for Alagille's syndrome, which produces cholestatis, pulmonic stenosis and poor school performance, among other deficits [70]. Recently, Hitoshi et al. demonstrated that **(a)** NSCs are missing almost completely in the E10.5 *Notch*$^{-/-}$ and E8.5 RBP-Jkappa$^{-/-}$ transgenic brains; **(b)** the number of NSCs in the *PS1*$^{-/-}$ brains decreases across embryogenesis; **(c)** the dissociation of single primary *PS1*$^{-/-}$ neurospheres from E14.5 brains produces fewer secondary neurospheres than do wild-type spheres. This decline of self-renewal ability of *PS1*$^{-/-}$ NCSs is partially rescued by transducing a constitutively active form of the *Notch1* gene, suggesting that diminished Notch signaling is responsible for the attenuated self-renewal of *PS1*$^{-/-}$ transgenic mouse NSCs [71]. Notch1, therefore, is a crucial regulator of neural stem cell maintenance and self-renewal during the development stage. Consistent with this finding, deletion of the basic helix-loop-helix (bHLH) transcriptional repressor *Hes1*, a known mediator of Notch signaling, causes premature neuronal progenitor cell differentiation and a reduction in the self-renewal capacity of embryonic forebrain NSCs [72]. Overexpression of activated Notch1 in the embryonic cortex results in an increase of radial glial cells [73], which have been implicated in neurogenesis [74].

Recently, a bevy of publications has alluded to the necessity of Notch signaling in the regulation of neurogenesis in the adult brain. Evidence for its involvement includes the following:

(1). Notch signaling components are expressed in neuroproliferative regions of the postnatal brain. In situ mRNA hybridization revealed that Notch1 is associated with cells

in the SVZ, DG and RMS [75], and that most Notch1-positive cells in the adult SVZ express PSA-NCAM and GFAP [76]. Notch1 is also found in PSA-NCAM-positive neuroblasts located within the RMS and, to a diminished extent, in those that have reached the OB. In addition, mRNA and protein for two of the Notch1 activators, Jagged1 and Delta1, are also expressed in the SVZ and the RMS in the adult brain [75, 76]. Downstream targets of the Notch1 signaling Hes1, Hes3 and Hes5, and the intrinsic Notch regulatory proteins Numb and Numblike are also associated with cells expressing Notch1 [75-77]. Notch1 was found to be mainly expressed in doublecortin (DCX)-positive cells corresponding to newborn neurons, whereas the Notch1 ligand, Jagged1, is predominantly expressed in GFAP-positive astrocytic cells in the SVZ of the normal adult brain [78]. These findings are confirmed by conditional depletion of DCX-positive cells in transgenic mice carrying herpes simplex virus thymidine kinase (HSV-TK) under the control of the DCX promoter [78].

(2). Notch1 expression in the SVZ is reduced with aging, in parallel with a reduction of neurogenesis [76].

(3). Disruption of Notch1 using antisense or a γ-secretase inhibitor demonstrated a requirement for Notch1 in the maintenance and proliferation of NSCs in the adult brain [79]. Ablation or overexpression of Notch1 in GFAP-expressing astroglial cells dramatically affects the proliferation, cell fate, and survival of progenitors as well as the maturation of newly generated neurons, displaying the central role of Notch1 in postnatal hippocampal plasticity [80]. *In vitro* studies show that blockage of the Notch pathway with DAPT not only significantly reduced the number of neurospheres, but also the diameter of spheres. In addition, attenuation of endogenous Notch with siRNA resulted in a significant reduction of the percentage of BrdU-positive cells compared with that in non-transfected ischemic neural progenitor cells [78].

(4). Transient administration of Notch ligands to the brain of adult rats increases the number of NSCs [81]. In addition, the astrogliogenic response of the SVZ to injury in a model of cortical stab wound is accompanied by activation of the Notch pathway [76]. These findings indicate that NSC expansion may be achieved by Notch ligands through a pathway that is fundamental to development and cancer [82].

Role of Notch signaling in neurogenesis after stroke

The NSCs located in neurogenic regions increase in response to stroke and neurodegenerative diseases [43, 83, 84], and newborn cells can migrate into damaged brain regions [85], where they differentiate into mature neuronal cells and integrate into local neuronal circuits. We and others find that the activated form of Notch1 (NICD) and its downstream transcriptional targets, Hes1, are also expressed in SVZ cells after ischemia [77] [86-88]. Increased activation of Notch1 signaling increases SVZ cell proliferation, whereas inhibiting Notch1 signaling resulted in a reduction of proliferating cells in the SVZ. Levels of NICD, Hes1, and Shh are increased in the SVZ at 4 and 24 hr after focal cerebral ischemia. Interestingly, ischemia-induced cell proliferation in the SVZ is blocked by inhibition of the Notch1 signaling pathway [77]. An in vitro study shows that blockage of the Notch signaling by siRNA against Notch or a γ-secretase inhibitor significantly reduces ischemia-mediated cell proliferation. During differentiation, Notch and Hes1 expression is down-regulated in neural progenitor cells after ischemia, which coincides with a significant increase in neuronal population, suggesting that the Notch signaling pathway mediates adult SVZ neural progenitor cell proliferation and differentiation after stroke [78]. These findings suggest that the Notch signaling pathway enhances the expansion of the neural progenitor pool and neuronal differentiation in adult neural progenitor cells after stroke.

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