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Review Article

Signaling pathways in cerebellar granule cells development

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Abstract: Cerebellar granule cells originate from precursors located at the dorsal region of rhombomere in the hindbrain of embryos. They undergo proliferation from embryo to post-natal period so as to form the major cell type of the cerebellum. The development of granule cell is not only highly dependent on the cerebellar intrinsic environment, but also is regulated by serials of transcription factors on different signaling pathways. Therefore, in this manuscript the signaling pathways participating in the proliferation and differentiation of granular cells during normal development was reviewed.

Keywords: Cerebellar granule cells, signaling pathway, development

Introduction

Cerebellar granule cells are the most abundant neurons in cerebellar neuronal circuitry. The granule cell precursors (GCPs) are generated from rostral hindbrain during late embryogenesis [1]. Expansion of GCPs pool takes place in the external granule layer (EGL) of cerebellum, with peak proliferation between post-natal day 5 and 8 in the mouse [2], but the mechanisms which control proliferation and differentiation of GCPs remain inadequately understood.

During the past years, great progresses have been attained in the identification of genes, particularly transcription factors, which mark the granule cell lineage from precursors to mature neurons. From the beginning of their proliferation in rhombic lip, granule cell precursors express a number of markers such as bHLH transcription factor, the zinc-finger transcription factor and genes implied in cell cycle. Post-natally, the granule cell precursors express notch2 and genes involved in Shh signaling pathway such as Ptc, Smo, gli1, gli2 and etc. [3, 4]. Signaling pathways that regulate the developmental process of cerebellar granule cells was reviewed in this manuscript.

The sonic hedgehog pathway

Regulation of cerebellar granule cell proliferation by Shh signaling

The hedgehog family, first identified in Drosophila embryonic developmental process, is highly conserved in mammals and includes three different proteins, sonic hedgehog, indian hedgehog and desert hedgehog. Shh was shown to play wide roles in various organs including nervous system during development. Moreover, Shh has been regarded as one of the master players in cerebellar patterning and was regarded as a key regulator of granule cell development [5, 6].

In the cerebellum, Shh is produced by Purkinje neurons and regulates the division of GCPs during post-natal development. Shh binds to the trans-membrane receptor Patched (Ptc) and relieves Ptc-mediated inhibition of Smoothened (Smo) activity. Smo, a G-protein-coupled receptor, activates an inhibitory G protein that leads to activation of Gli transcription factors and the initiation of gene expression required for cell cycle progression. However, the Shh signaling intermediates that regulated GCP proliferation are just beginning to be understood [3, 5, 7].

The important role played by Shh in GCP proliferation has been linked directly to cell cycle, through which Shh induces expression of D cyclins during development via N-myc. N-myc is a member of the Myc/Max/Mad family of basic helix-loop-helix leucine zipper (bHLHZ) DNA binding proteins that plays function in most instances as a transcriptional activator. Both Myc and Mad proteins form heterodimers with the cofactor Max, thereby permitting binding to specific DNA motifs known as E-box sequences. These DNA-bound heterodimers recruit co-activator or co-repressor complexes that generate alterations in chromatin structure and transcriptional activity. For example, Mad3 interacts with Max and the mSin3 co-repressor to repress transcription from a reporter promoter containing an E-box CACGTG sequence in cultured fibroblasts [8, 9].

In the cerebellum, *N-myc* is expressed in proliferating GCPs during the clonal expansion phase in vivo and is up-regulated in GCPs in response to Shh treatment in vitro. Furthermore, over-expression of N-myc in cultured GCPs leads to an increase in proliferation and the expression of D cyclins. Conversely, inactivation of *N-myc* in neural progenitor cells in vivo leads to a smaller and disorganized cerebellum with a reduced cell density in internal granule layer. Through a microarray-based approach, genes transiently up-regulated during GCP proliferation with profiles similar to those of known Shh target genes such as cyclin D2 were identified. Among which, it was indicated that Mad3 is a member of the Mad family of bHLHZ transcriptional regulators comprised of Mad1, Mxi1, Mad3, and Mad4 [8, 10]. Mad proteins are part of the larger family of Max binding proteins to which N-myc also belongs. Mad family members have been shown to inhibit cell cycle progression and promote differentiation [11].

The expression profile of Mad3 correlated highly with that of cyclin D2 and Mad3 failed to be down-regulated in weaver mice compared with wild-type littermates. Cerebellar granule cells in weaver mice fail to switch off the cell cycle and differentiate, suggesting that Mad3 may play a role in cell cycle progression of GCPs. Indeed, the expression profile of cyclin D2 displayed a pattern in wild-type and weaver mice which were similar to that of Mad3. Because the products of genes with similar expression profiles have been shown to function in the same pathway, these data suggested that Mad3 might be a component of the Shh pathway in the cerebellum. There is evidence to support a novel role for Mad3 in the Shh pathway to promote proliferation of cerebellar GCPs. It is demonstrate that Mad3 is necessary for Shh-mediated proliferation by highly purified cultures of GCPs. Furthermore, over-expression of Mad3 is sufficient to induce GCP proliferation in the absence of Shh. Structure function analysis revealed that dimerization with Max and recruitment of the Sin3 co-repressor is required for Mad3-mediated GCP proliferation. Surprisingly, DNA binding via the basic domain of Mad3 is not required, suggesting that Mad3 interacts with other DNA binding proteins to repress transcription and challenge the current paradigm that Mad3 should antagonize N-myc by competition for direct DNA binding via Max dimerization [5, 12, 13].

Further study indicated that Shh not only functions as a GCP mitogen in vitro, but also influences foliation during cerebellum development by regulating the position and/or size of lobes. It demonstrated that Shh expression correlates spatially and temporally with foliation. Expression of the Shh target gene Gli1 is also highest in the anterior medial cerebellum, but is restricted to proliferating GCPs and Bergmann glia. By contrast, Gli2 is expressed uniformly in all cells in the developing cerebellum except Purkinje cells and Gli3 is broadly expressed along the anteroposterior axis. Whereas Gli mutants have a normal cerebellum, Gli2 mutants have greatly reduced foliation at birth and a decrease in GCPs. In a complementary study using transgenic mice, it was identified that over-expressing Shh in the normal domain does not grossly alter the basic foliation pattern, but does lead to prolonged proliferation of GCPs and an increase in the overall size of the cerebellum [14, 15].

The regulation of Shh signaling pathway

Committed EGL granule cell precursors require Shh signaling for proliferation, which at least in mice could be driven first by transient autocrine Shh signaling and then by Shh from Purkinje neurons. Shh protein could be released from Purkinje cell bodies or dendrites, the latter representing a possible parallel with the inducing

action of Hedgehog from fly retinal axons prematurely [16]. In vitro studies provide evidence that β1 integrins act at least in part cell autonomously in GCPs to regulate their proliferation. Previous studies have shown that sonic hedgehog (Shh)-induced GCP proliferation is potentiated by the integrin ligand laminin. It was indentified Shh directly binds to laminin and that laminin-Shh induced cell proliferation is dependent on β1 integrin expression in GCPs. An important finding is that β1 integrins, their LN ligands and Shh cooperate to regulate GCP proliferation. Shh is the most potent promoter of GCP proliferation, which not only confirm that the proliferative responses to Shh are potentiated by laminin-1 (LN-1), but also show that Shh binds to LN-1 and that expression of β1 integrins in GCPs is required for LN-1 to enhance GCP proliferation. These data are consistent with a model where β1 integrins help to recruit Shh-LN-1 complexes to the cell surface of GCPs, increasing the apparent concentration of Shh close to the Patched-Smoothened receptor complex. However, binding to LN-1 alone cannot be sufficient, because Shh also binds to VN, but VN appears to induce differentiation. The results rather are consistent with a model in which β1 integrins not only help to recruit Shh-LN-1 complexes to the cell surface, but also regulate the activity of second messenger systems more directly [17, 18].

Notch signaling pathway in cerebellar cells development

Notch is a transmembrane molecule which functions as a receptor influencing cell fate decision in various organs. In mammals, four Notch molecules (Notch1-4) and five ligands (Delta-like (Dll)1, 3 and 4 and Jagged1 and 2) have been identified to date. In the central nervous system, Notch1-3 are expressed in stem and progenitor cells in embryonic animals, suggesting their functions in neurogenesis and gliogenesis [19, 20]. The activation of Notch signaling is initiated by the direct interaction of ligands, such as Delta or Jagged, which leads to the activation of the RBP-J-dependent and RBP-J independent pathways (Deltex-dependent pathways). In the classical RBP-J-dependent pathways, the intracellular portion of Notch (NICD) is cleaved upon ligand binding and enters the nucleus, where it forms a complex with RBP-J and influences the expression of numerous transcription factors. On the other hand, the cytoplasmic protein (termed "Deltex") is essential for RBP-J-independent signaling. It has been demonstrated that Deltex regulates Notch signaling by physically interacting with NICD independently of RBP-J, although the molecular basis of this Deltex-dependent signaling is largely unknown [21, 22].

Several previous studies showed that Notch2 is expressed by granule cell precursors at EGL in the postnatal cerebellum. Solecki further showed that Jagged1-Notch2 signaling inhibits differentiation of granule cell precursors in vitro. The Notch pathway appears to promote GCP proliferation and prevent differentiation. At E16.5, the Notch2 receptor is expressed at low levels in the developing cerebellum. Its expression is greatly increased around the peak of the clonal expansion period in EGL but also in Bergmann glia, before declining again as the granule cells differentiate [21, 23]. Treatment of GCPs in vitro with a soluble form of the Notch ligand Jagged, has a similar mitogenic effect as Shh at low concentrations, resulting in Notch pathway activation. While constitutive activation of the Notch pathway in P6 organotypic cerebellar slices by over-expression of Notch2 results in inhibition of granule cell neurite extension. These findings place Notch signaling at the heart of the proliferation/differentiation switch in GCPs. Conditional inactivation of Notch1, within the cerebellar primordium at 10 dpc using the En2-Cre line, results in overall cerebellar hypoplasia in the adult. This is thought to be due to an early onset of differentiation but also increased apoptosis affecting the Purkinje cells [24, 25].

Wnt signaling in cerebellar cells development

The Wnt signaling pathway is an autocrine-paracrine signal transduction pathway. Its signaling has been demonstrated to participate in axis formation, midbrain development and oncogenesis [27]. Increased Wnt signalling has been detected in both murine and human Medulloblastoma (MB) tumours. Wnt signalling is crucial for early cerebellar specification, patterning and synaptogenesis in the cerebellum [28, 29]. However, a possible role in GCP proliferation and/or differentiation has not been well established. A study involving lithium chloride treatment, which acts as a Wnt pathway ago-

nist, reported a positive effect on GCP proliferation in vitro. Nestin-Cre mediated conditional inactivation of β-catenin, an intracellular component of the Wnt pathway which regulates gene expression in combination with other transcription factors in response to Wnt signalling, results in vermis hypoplasia and early neuronal commitment but not complete differentiation of cerebellar progenitors at 14.5 during development. Despite this, the major neuronal cell types, including the granule cells, do differentiate in these mice, highlighting an important role for the Wnt pathway in early GCP proliferation/differentiation switch. Furthermore, lack of the Frizzled4 receptor results in granule cell and Purkinje cell degeneration, emphasizing a requirement for the Wnt pathway in the maintenance of granule cells in the adult [30-32].

Recent studies suggest that Wnt factors play a role in the formation of neuronal connection. First, overexprssion of the Drosophila Wnt gene, DWnt-3, encoding a protein localized to axonal processes, disrupts commissural axon tracts. Second, several mouse Wnt genes are expressed in post-mitotic neurons during periods of axonal extension and synaptogenesis. Third, Wnt-7a, which is expressed by cerebellar GCs, induces axonal remodeling in cerebellar granule neurons in vitro, a process characterized by shortening of the axon, axonal spreading, axonal branching, and increased growth cone size. Wnt-2a induces axonal remodeling by inhibiting the activity of GSK-3β, a kinase highly expressed in the CNS [33]. GSK-3β directly phosphorylates several microtubule-associated proteins such as Tau, MAP-1B and MAP-2. Wnt-7a induces a decrease in the GSK-3β phosphorylated form of MAP-1B with a concomitant unbunding of stable microtubules from spread areas of the axon. Subsequently, Wnt-7a increases the level of synapsin I at these spread areas, a process also mediated by GSK-3β activity. These findings suggest that Wnt-7a, by regulating GSK-3β activity, induces axonal remodeling and the initiation of synaptogenesis during cerebellar development [34, 35].

Although the three signaling pathways mentioned above all take part in the regulation of proliferation and differentiation during cerebellar granule cells development, few data indicated that Shh and Wnt work in opposition across the dorso-ventral axis of the cerebellum to regulate formation of $olig^{2+}$ neurons [36]. It was presumed that Shh limit the range of Wnt signaling, which is necessary for $olig^{2+}$ neuron development. However, the interaction of which is not known yet. Maybe the different signaling pathways play different roles in different phases of granule cells development. Whatever the case, much work remains to be done to answer these questions.

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Disclosure of conflict of interest

None.

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