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The scaffold protein JSAP1 regulates proliferation and differentiation of cerebellar granule cell precursors by modulating JNK signaling

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ABSTRACT

Cerebellar granule cell precursors (GCPs) proliferate in the outer part of the external granular layer (EGL). They begin their differentiation by exiting the cell cycle and migrating into the inner part of the EGL. Here we report that JSAP1, a scaffold protein for JNK signaling pathways, is expressed predominantly in the post-mitotic GCPs of the inner EGL. JSAP1 knockdown or treatment with a JNK inhibitor enhances the proliferation of cultured GCPs, but the overexpression of wild-type JSAP1 leads to increased proportions of $p27^{Kip1}$- and NeuN-positive cells, even with saturating concentrations of Sonic hedgehog (Shh), a potent GCP mitogen. However, these differentiation-promoting effects on GCPs are attenuated significantly in cells overexpressing a mutant JSAP1 that lacks the JNK-binding domain. Together, these data suggest that JSAP1 antagonizes the mitogenic effect of Shh on GCPs and promotes their exit from the cell cycle and differentiation, by modulating JNK activity.

Introduction

Cerebellar granule cells, which are by far the most abundant neurons in the central nervous system, arise from a germinal zone in the rhombic lip. The granule cell precursors (GCPs) leave the rhombic lip and migrate tangentially to form a secondary germinal zone, called the external granular layer (EGL). In rodents, GCPs in the outer part of the EGL proliferate extensively for 2–3 weeks after birth, thus greatly expanding their population. GCPs start differentiating by exiting the cell cycle and moving into the inner part of the EGL. They then migrate further and radially past the Purkinje cells to their final destination, the internal granular layer (IGL) (Hatten, 1999).

Previous studies have indicated that multiple molecules act as mitogenic factors for GCPs, of which sonic hedgehog (Shh) is likely to be the most potent (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). On the other hand, Miyazawa et al. (2000) demonstrated that $p27^{Kip1}$, a cyclin-dependent kinase (CDK) inhibitor, is involved in terminating the division of GCPs and permitting their differentiation. Furthermore, basic fibroblast growth factor (bFGF) has been reported to block Shh signaling in GCPs and to promote their differentiation by modulating c-Jun NH_2-terminal kinase (JNK) activity (Wechsler-Reya and Scott, 1999; Fogarty et al., 2007). Together, these studies have shed considerable light on the molecules involved in determining the fate of GCPs, in terms of their decision to continue or stop dividing. However, it is largely unknown how intracellular signaling pathways are activated specifically and efficiently to exert the relevant cell-fate specification of GCPs.

Mammalian mitogen-activated protein kinase (MAPK) intracellular signaling pathways play key roles in multiple cellular processes, including proliferation and differentiation (Kyriakis and Avruch, 2001; Chang and Karin, 2001). Scaffold proteins for MAPK pathways are thought to function in the spatial and temporal regulation of these pathways by organizing the MAPK signaling components into functional modules (Morrison and Davis, 2003; Yoshioka, 2004; Dhanasekaran, et al., 2007). These scaffolding complexes enable the efficient activation of specific MAPK cascades. We previously identified JNK/stress-activated protein kinase-associated protein 1 (JSAP1, also known as JNK-interacting protein 3 (JIP3)) as a scaffold protein for mammalian JNK MAPK pathways (Ito et al., 1999, 2000; Kelkar et al., 2000). In the developing mouse brain, JSAP1 is highly expressed in progenitor cells, including the GCPs of the cerebellar EGL (Miura et al., 2006). In the present study, we analyzed the expression of JSAP1 and JNK in the developing mouse cerebellum by immunohistochemistry. We also studied the proliferation and differentiation of cultured GCPs in which we knocked down the RNA for JSAP1 or forced the expression of either wild-type JSAP1 or a mutant JSAP1 lacking its JNK-binding domain (JBD), in the presence or absence of Shh. Our results strongly suggest that JSAP1–JNK signaling promotes the cell-cycle exit and differentiation of cerebellar GCPs.

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Results

Expression of JSAP1 and JNK in the developing cerebellum

To determine if JSAP1 could play a role in GCP fate decisions, we first examined the expression of JSAP1 and JNK in the developing mouse cerebellum by immunohistochemistry (Figs. 1 and 2; Supplementary Fig. 1). We double-labeled frozen sections of postnatal day 6 (P6) mouse cerebella with antibodies against JSAP1, JNK, phosphorylated (activated) JNK (P-JNK), Ki67 (a proliferation marker), $p27^{Kip1}$ (a negative regulator of the GCP cell cycle; Miyazawa et al., 2000), NeuN (a neural differentiation marker), and calbindin (a Purkinje cell marker), focusing on their expression patterns in the EGL. The proteins double-labeled with JSAP1 were Ki67, $p27^{Kip1}$, NeuN (Fig. 1), JNK, P-JNK (Fig. 2), and calbindin (Supplementary Fig. 1). In addition, double immunostaining for P-JNK and calbindin was performed (Supplementary Fig. 1). For these double staining experiments, two types of antibodies against P-JNK, i.e. monoclonal and polyclonal antibodies, were used: the former in Fig. 2 and the latter in Supplementary Fig. 1. The specificity of both antibodies was confirmed by immunoblotting, in which mouse embryonic fibroblasts (MEFs) were stimulated to activate JNK, and the cell lysates were untreated or treated with bacterial alkaline phosphatase (BAP) prior to immunoblotting (data not shown). Ki67, NeuN, and $p27^{Kip1}$ are known nuclear proteins; JSAP1, JNK, and P-JNK were, by contrast, expressed exclusively in the cytoplasm.

As expected, the outer EGL was positive for the proliferating cell marker Ki67, and the inner EGL was positive for $p27^{Kip1}$ and NeuN (Figs. 1B, E, H). Labeling for JSAP1 was low to moderate in the outer EGL and intense in the inner EGL. The immunofluorescent signals for JNK showed a very similar pattern and mostly overlapped with the JSAP1 labeling (Figs. 2C, G). There was little immunoreactivity for P-JNK in the outer EGL or in the most superficial portion of the inner EGL, but P-JNK staining was intense in the lower portion of the inner EGL (Figs. 2M, O). Thus, most cells in the inner EGL that expressed P-JNK strongly were also JSAP1-positive. In addition, just superficial to the area showing strong P-JNK labeling, there was a thin stripe of cells that were positive for JSAP1, but expressed P-JNK at low levels. Finally, calbindin immunolabeling was observed in cells beneath the EGL, as expected for a Purkinje cell marker, and some calbindin-positive cells were co-labeled by antibodies to JSAP1 and P-JNK (Supplementary Fig. 1).

Together, these observations indicate that JSAP1-JNK signaling takes place at the appropriate time and place to play important roles in promoting GCP cell-cycle exit and differentiation.

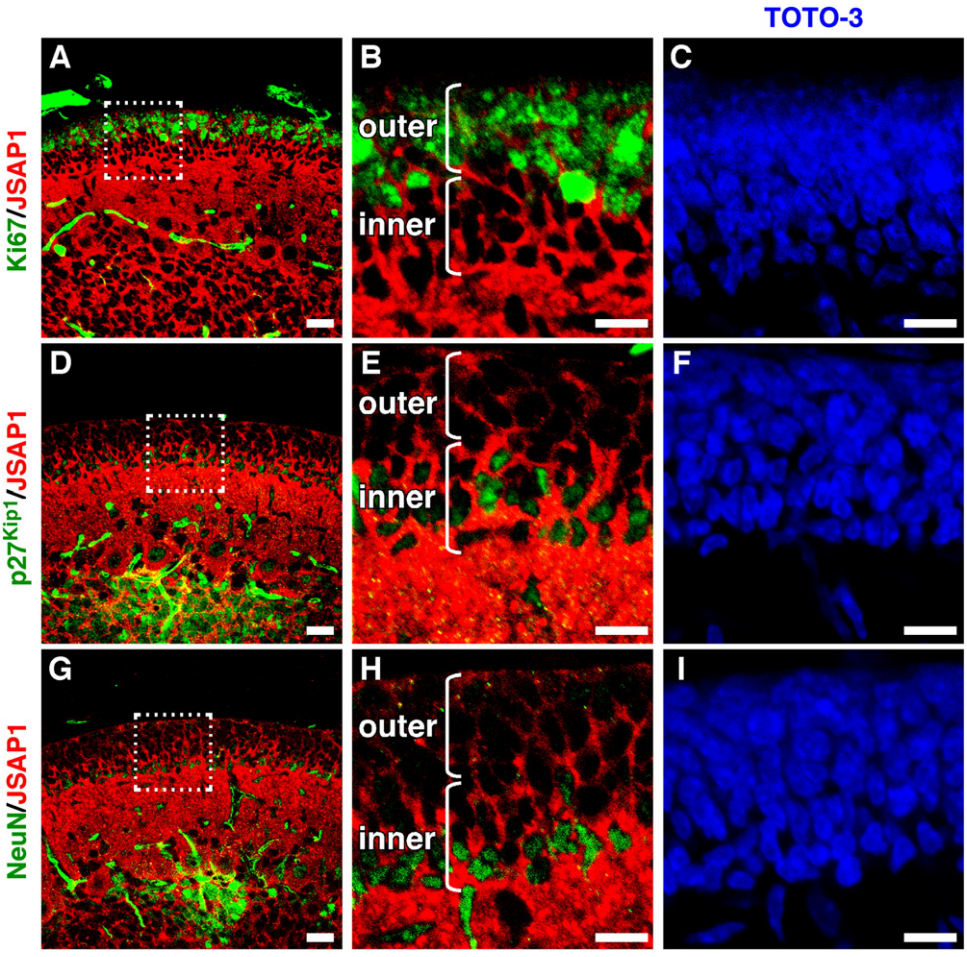

Fig. 1. Expression of JSAP1 and marker proteins in developing mouse cerebellum. Sagittal sections from P6 mouse cerebella double labeled with anti-JSAP1 (red) and (in green) anti-Ki67 (A, B), anti-$p27^{Kip1}$ (D, E), or anti-NeuN (G, H). Ki67, $p27^{Kip1}$, and NeuN are markers for proliferating, post-mitotic, and differentiating cells, respectively. B, E, and H show high-magnification views of the areas indicated in A, D, and G, respectively. C, F, and I, show nuclei stained with TOTO-3 (blue). Cells within the EGL and outside the EGL were positive for JSAP1 immunolabeling. Intense labeling for JSAP1 was detected in the inner EGL, which is a $p27^{Kip1}$- and NeuN-positive, but Ki67-negative post-mitotic zone. By contrast, moderate or low levels JSAP1 were detected in the outer EGL, which was Ki67-positive, but $p27^{Kip1}$- and NeuN-negative; i.e., a proliferation zone. outer, outer EGL; inner, inner EGL. Scale bars: 20 μm in A, D and G; 10 μm in B, C, E, F, H, and I. Images were captured with a confocal microscope.