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メタデータ	言語: eng 出版者: 公開日: 2017-10-03 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	http://hdl.handle.net/2297/12042

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

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Running title: JSAP1-JNK signaling regulates GCP development in cerebellum

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Number of figures and tables: 9 figures, 2 supplementary figures, and 0 tables

Number of pages (including figures): 45 pages

Number of words in Abstract: 146

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 NH₂-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.

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 (Fig. 1, Fig. 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 double-labeled proteins with JSAP1 were Ki67, p27^{Kip1}, NeuN (Fig. 1), JNK, P-JNK (Fig. 2), and calbindin (Supplementary Fig. 1). In addition, the 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 later in Supplementary Fig. 1, respectively. 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 the 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.

JSAP1 loss-of-function enhances the proliferation of cultured GCPs

To determine whether JSAP1 activity is necessary and sufficient for regulating the proliferation and differentiation of GCPs, we used cultured GCPs purified from the cerebella of P4 mice, and analyzed their expression of various markers by immunohistochemistry.

First, we characterized Ki67 and p27^{Kip1} expression in the primary culture system 24 h and 72 h after plating (Fig. 3), in the presence or absence of Shh. In the absence of Shh, the proportion of Ki67-positive proliferating cells decreased over time (44% and 27% in cells cultured for 24 h and 72 h, respectively), but the percentage of cells expressing p27^{Kip1} increased (50% and 71% in cells cultured for 24 h and 72 h, respectively). Incubation with Shh increased the GCP proliferation significantly at 24 h (62%) and 72 h (39%). Concomitantly, the proportion of p27^{Kip1}-positive cells observed was lower (29% at 24 h and 54% at 72 h). These results are essentially consistent with those reported previously (Miyazawa et al., 2000). In addition, there were very few apoptotic cells or

glial fibrillary acidic protein-positive glial cells in the 72-h culture (data not shown).

To learn whether JSAP1 functions in the control of GCP proliferation and differentiation, we used an RNA interference (RNAi) approach. First, we examined the effectiveness of an shRNA specific for JSAP1 (JSAP1-shRNA) (Fig. 4A). The JSAP1-shRNA stem sequence is identical to, but 2 bases shorter than, that of JSAP1-shRNA1, the most effective of four shRNAs reported previously (Bayarsaikhan et al., 2007). Transient co-expression of JSAP1-shRNA with Flag-tagged JSAP1 in human embryo kidney 293T (HEK293T) cells almost completely suppressed the expression of Flag-JSAP1, confirming the effective silencing of JSAP1 expression by JSAP1-shRNA. Next, GCP cultures were infected with lentiviruses expressing green fluorescent protein (GFP) plus either a control shRNA or JSAP1-shRNA. The JSAP1-shRNA substantially suppressed the expression of endogenous JSAP1 (Fig. 4B). We evaluated the effect of JSAP1 on GCP proliferation by counting the GFP-positive GCPs that incorporated bromodeoxuridine (BrdU) (Fig. 4C). The proportion of JSAP1-shRNA-infected GCPs in S phase increased significantly compared with that in the control shRNA-infected GCPs (Fig. 4D). Concomitantly, the population of p27^{Kip1}- and NeuN-positive cells significantly decreased in the JSAP1-shRNA-infected GCPs (data not shown). Together, these results suggest that JSAP1 functions to inhibit the proliferation of GCPs and to promote their differentiation.

Inhibition of JNK activity enhances the proliferation of cultured GCPs

We next asked whether JNK also acts as a negative regulator of GCP proliferation, because JSAP1 is a scaffolding protein for JNK cascades (Ito et al., 1999; Kelkar et al., 2000). To this end, we analyzed the effect of JNK inhibition on the proliferation of cultured GCPs. We first tested if overexpression of a fusion protein of the JSAP1 JBD with GFP (GFP-JBD) would inhibit the activity of JNK in HEK293T cells (Fig. 5A).

HEK293T cells transiently transfected with expression plasmids for either Flag-tagged GFP or Flag-GFP-JBD were stimulated by UV to activate JNK. The phosphorylation of the transcription factor c-Jun, a bona fide substrate for JNK, was analyzed by immunoblotting using an anti-phospho-c-Jun-specific antibody. The phosphorylation level of the endogenous c-Jun was markedly reduced in cells expressing Flag-GFP-JBD compared with those expressing Flag-GFP, indicating that the fusion protein was an effective JNK inhibitor.

Next, GCP cultures were infected with lentiviruses expressing either Flag-GFP or Flag-GFP-JBD, and GCP proliferation was assessed by the incorporation of BrdU by GFP-positive GCPs (Figs. 5B, C). The proportion of the proliferating cells among Flag-GFP-JBD-infected GCPs significantly increased with respect to the control Flag-GFP-infected GCPs, and the population of p27^{Kip1}- and NeuN-positive cells significantly decreased (data not shown). Together, these observations suggest that JNK, like JSAP1, negatively regulates GCP proliferation and promotes GCP differentiation.

JSAP1-JNK signaling promotes the cell-cycle exit and differentiation of primary cultured GCPs

To determine whether JSAP1 promotes the cell-cycle exit and differentiation of GCPs in association with JNK, we overexpressed wild-type JSAP1 or its mutant lacking the JBD in cultured GCPs in the presence or absence of Shh, and evaluated the effects by immunocytochemistry with antibodies against p27^{Kip1} and NeuN, and by BrdU incorporation (Figs. 6 and 7).

We confirmed that the hemagglutinin (HA)-tagged wild-type and mutant JSAP1 proteins, HA-JSAP1(WT) and HA-JSAP1(Δ JBD), respectively, were expressed at similar levels in transiently transfected HEK293T cells (Fig. 6A). In the absence of Shh, the lentivirus-mediated overexpression of HA-JSAP1(WT) led to a significant increase in the

proportion of p27^{Kip1}-positive cells in the GCP cultures compared with control lentivirus-infected cultures (Fig. 6B). Concomitantly, a significant decrease in the proportion of BrdU-positive cells was detected among the HA-JSAP1(WT)-infected GCPs (Fig. 6C). In contrast, the respective increase and decrease in the proportions of p27^{Kip1}- and BrdU-positive cells seen with the expression of HA-JSAP1 were significantly smaller in the JSAP1(Δ JBD)-infected GCPs (Figs. 6B, C).

We further studied whether overexpressed HA-JSAP1(WT) could exert its effects on GCPs even in the presence of Shh. Although Shh treatment lowered the proportion of p27^{Kip1}-positive cells and increased the proportion of BrdU-positive cells in the control cultures, in GCP cultures overexpressing HA-JSAP1(WT), the percentage of p27^{Kip1}-positive cells increased and that of the BrdU-positive cells decreased significantly, compared with control lentivirus-infected GCPs (Figs. 6B, C). When HA-JSAP1(Δ JBD) was expressed instead of HA-JSAP1(WT), the respective increase and decrease in the proportions of p27^{Kip1}- and BrdU-positive cells were significantly attenuated (Fig. 6B, C). We also examined the effects of overexpressed HA-JSAP1(WT) or HA-JSAP1(Δ JBD) on the differentiation of GCPs in culture, as measured by labeling with the anti-NeuN antibody. The results were very similar to those obtained with the anti-p27^{Kip1} antibody (Fig. 7).

To confirm the effect of JSAP1 on GCP differentiation, we examined the expression of a subset of markers for postmitotic neuroblasts, *Neurod1*, *Nfia*, and *Pax6* (Morales and Hatten, 2006; Schüller et al., 2006; Wang et al., 2007), by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis (Fig. 8). We coexpressed HA-JSAP1(WT) or HA-JSAP1(Δ JBD) with GFP in GCP cultures, and GFP-positive cells were purified by fluorescence-activated cell sorting (FACS). Total RNAs were then prepared, and subjected to real-time RT-PCR analysis using specific primers for the markers. As shown in Figure 8, the expression levels of all the markers examined were significantly higher in cells expressing HA-JSAP1(WT) compared with those expressing

HA-JSAP1(Δ JBD). Furthermore, when JNK activity was measured using a luciferase reporter system in cultured GCPs transiently expressing HA-JSAP1(WT) (Fig. 9), the JNK activity was found to increase dose-dependently with the amount of the expression plasmid used for transfection. When HA-JSAP1(Δ JBD) was used instead of HA-JSAP1(WT), the increase in JNK activity was significantly less, although it was not entirely abrogated.

Together, these observations strongly suggest that JSAP1 antagonizes the inhibitory effect of Shh on GCP differentiation, promoting GCP exit from the cell cycle and differentiation, by modulating JNK activity.

Discussion

In the present study we analyzed the role of JSAP1, a scaffold protein for JNK signaling pathways, in mouse cerebellar GCPs. We propose that JSAP1 antagonizes the mitogenic effect of Shh on GCPs, and promotes the exit of GCPs from the cell cycle and their differentiation by modulating JNK activity in the developing cerebellum. The following lines of evidence support this proposal: both JSAP1 and P-JNK are expressed predominately in non-dividing GCPs in the developing cerebellum; JSAP1 knockdown or treatment with a JNK inhibitor enhances the proliferation and suppresses the differentiation of cultured GCPs; the expression of exogenous wild-type JSAP1 in cultured GCPs leads to increased numbers of p27^{Kip1}- and NeuN-positive cells with a concomitant decrease in the proportions of BrdU-positive cells, even in the presence of saturating concentrations of Shh; and the expression of a subset of markers for postmitotic neuroblasts is increased in cultured GCPs expressing exogenous wild-type JSAP1. Furthermore, all of these differentiation-promoting effects on GCPs are significantly diminished in cells expressing a mutant JSAP1 that lacks the JBD.

It seems likely that JSAP1 signaling complexes receive extracellular signals through transmembrane proteins, because JSAP1 is, at least in part, a plasma membrane-associated protein (Cavalli et al. 2005; Miura et al., 2006). However, to date, the physiologically relevant receptors that transmit signals to JSAP1 complexes remain largely unknown. Wechsler-Reya and Scott (1999) and Fogarty et al. (2007) reported that bFGF blocks Shh signaling and promotes the differentiation of GCPs. Considering that bFGF and the JSAP1-JNK signaling pathway have similar effects on GCPs, JSAP1 scaffolding complexes seem likely to act downstream of FGF receptors in GCPs to switch the cells from proliferation to differentiation. In addition, extracellular matrix (ECM) glycoproteins, such as laminin (LN), vitronectin (VN), and proteoglycans, have been shown to regulate the development of GCPs (Pons et al., 2001; Rubin et al., 2002; Blaess et al., 2004). Pons

et al. (2001) suggested that the ECM glycoprotein VN, but not LN, acts to terminate Shh-mediated proliferation and induce the differentiation of GCPs in the developing cerebellum. Thus, it is possible that the JSAP1-JNK pathways are involved in VN-specific integrin receptor-mediated signaling.

The cerebellar GCPs proliferate massively in the EGL and migrate along an inward radial path to the IGL. We showed here that the JSAP1 protein is expressed in cells not only within the EGL, but also beneath it. In addition to the calbindin-positive cells, i.e. the Purkinje cells, a subset of the JSAP1-positive cells found beneath the EGL is likely to consist of migrating GCPs. We previously reported that JSAP1 cooperates with focal adhesion kinase (FAK) to regulate JNK activity and cell migration in cultured cells (Takino et al., 2002, 2005). Hirai et al. (2002, 2006) demonstrated that dual leucine zipper kinase (DLK), an upstream kinase in JNK cascades, regulates axon projection and neuronal migration in the developing cerebral cortex. In addition, DLK is expressed in the developing cerebellum as well (Suenaga et al., 2006). Together, these observations suggest that the scaffolding protein JSAP1 may be involved in the coordinated regulation of the axon growth and radial migration of GCPs, by organizing the DKL-JNK pathway in collaboration with signaling molecules such as FAK.

The immunohistochemical analysis in the present study showed that the expression profile of JNK is quite similar to that of JSAP1 in P6 mice. Furthermore, the expression patterns for high levels of P-JNK and for JSAP1 were largely overlapping, although JSAP1 was also expressed in a marginally more superficial region of the inner EGL of the developing cerebellum where P-JNK expression was low. Previous biochemical studies by our group and others have strongly suggested that JSAP1 regulates JNK cascades as a scaffolding factor (Ito et al., 1999; Kelkar et al., 2000). Furthermore, Byrd et al. (2001) established by genetics that a functional relationship exists between UNC-16, the ortholog of JSAP1 in *C. elegans*, and JNK signaling. Together, these findings suggest that the slight positional difference we observed for these molecules in the developing cerebellum may

reflect the mode of JNK activation in GCPs. That is, during the cell-cycle exit step, JNK bound to JSAP1 is activated as P-JNK, and the scaffold protein-mediated activation of JNK in turn triggers the cessation of GCP proliferation, leading to the movement of the post-mitotic GCPs into the premigratory zone, the inner part of EGL. This sequence of events would produce the immunohistochemical reaction patterns that we observed.

In mammals, there are three *Jnk* genes, *Jnk1*, *Jnk2*, and *Jnk3*. We have recently shown that all the *Jnk* genes are expressed in developing mouse brain by *in situ* hybridization (Miura et al., 2006). Here we investigated the expression of these *Jnk* mRNAs in GCPs prepared from P4 mouse cerebellum by quantitative real-time RT-PCR analysis, and found that the expression level of *Jnk3* was highest among the *Jnk* genes in the GCPs (Supplementary Fig. 2). In addition, our group and others previously reported that JNK3 showed higher binding affinity to JSAP1 than did JNK1 and JNK2 (Ito et al., 1999; Kelkar et al., 2000). Taken together, it is likely that JSAP1-JNK3 signaling would be mainly responsible for the regulation of GCP differentiation in the developing cerebellum. However, as JNK1 and JNK2, but not p38 α and extracellular signal-regulated kinase 2 MAPKs, could also bind to JSAP1 at least in coexpressed cultured cells (Ito et al., 1999; Kelkar et al., 2000) and JNK1/2 proteins are likely to be expressed in GCPs, we cannot rule out the possibility that all the JNK isoforms are involved in regulating the exit of GCPs from the cell cycle and their differentiation.

Here we found that the forced expression of wild-type JSAP1 in cultured GCPs increased the percentages of p27^{Kip1}- and NeuN-positive GCPs, and the population of BrdU-positive GCPs decreased concomitantly, and that these effects were significantly less strong when a mutant JSAP1 lacking the JBD was expressed instead of the wild-type JSAP1. Furthermore, we monitored the JNK activity by a luciferase reporter system in GCPs expressing wild-type JSAP1, and found enhanced JNK activity in these cells. Again, when the mutant JSAP1 lacking the JBD was used instead of the wild-type JSAP1, the enhanced JNK activity was substantially suppressed, although not completely abolished.

Because JSAP1 protein forms an oligomeric complex, at least when transiently overexpressed (Kelkar et al., 2000; our unpublished observation), a portion of the exogenous JSAP1 mutant might associate with the endogenous wild-type JSAP1. The heteromeric JSAP1 complexes may be functionally active, as in the case for the yeast scaffold protein Ste5 (Inoue et al., 1997), in terms of JNK activation. Alternatively, there may exist one or more additional signaling pathways that regulate GCP proliferation and/or differentiation and are independent of the JSAP1-JNK pathways.

The CDK inhibitor p27^{Kip1} participates in stopping the proliferation of neural progenitors (Casaccia-Bonofil et al., 1999; Miyazawa et al., 2000; Goto et al., 2004). Although the mechanism by which the expression of p27^{Kip1} is controlled in GCPs remains unclear, JNK organized by the scaffold protein JSAP1 may be directly involved in its regulation. Recently, Kawauchi et al. (2006) demonstrated that cyclin-dependent kinase 5 (CDK5), a proline-directed serine/threonine kinase, phosphorylates and stabilizes p27^{Kip1} in cortical neurons. Because JNK is also a proline-directed serine/threonine kinase, JNK may fulfill this role instead of CDK5 in GCPs. It is also possible that the JSAP1-JNK pathways positively regulate the expression of p27^{Kip1} at the transcriptional level by modulating the activities of transcription factors. Further investigation will be necessary to clarify this issue.

An important finding of this study is that a scaffold protein for JNK cascades, JSAP1, inhibits the Shh-induced proliferation of cultured GCPs. The deficient down-regulation of proliferation in cerebellar GCPs is thought to give rise to medulloblastoma, the most common and aggressive brain malignancy of childhood. Di Marcotullio et al. (2004) identified REN (also known as KCTD11) as a putative tumor suppressor, the locus of which is frequently deleted in human medulloblastoma. Interestingly, REN shows an expression profile similar to that of JSAP1 in the EGL of the developing cerebellum, and furthermore, antagonizes Shh signaling in cultured GCPs (Di Marcotullio et al., 2004; Argenti et al., 2005). The JSAP1-JNK pathways therefore may function in the control of

REN expression in GCPs during cerebellar development.

In summary, our findings suggest that the JSAP1-JNK signaling pathways regulate cellular programs to switch from proliferation to differentiation in cerebellar GCPs. This study sheds new light on the scaffolding protein-mediated mechanisms by which specific signaling pathways are activated and regulated spatio-temporally to determine the cell fate of neural precursors.

Experimental methods

Animals

All the experiments involving animals were conducted according to the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan. C57BL/6J mice were purchased from SLC (Hamamatsu, Japan).

BrdU labeling and detection

For BrdU labeling, BrdU (Sigma, St. Louis, MO) was added to cultured GCPs at a concentration of 10 μ M, 12 h before fixation, and the GCPs were processed for immunocytochemistry as described below. A mouse monoclonal anti-BrdU antibody (1:100; BD Biosciences, San Jose, CA) was used to detect BrdU.

Immunohistochemistry

Mice were deeply anesthetized and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PB) pH 7.4. The entire brain was removed and postfixed overnight at 4°C, cryoprotected in 30% sucrose, and embedded in OTC compound. Sections (25- μ m thick) were subjected to conventional double immunofluorescence by incubation for 1 h at room temperature with 2% BSA, 2% goat serum, and 0.4% Triton X-100 in Tris-buffered saline (TBS) pH 7.4 for blocking, followed by overnight incubation at 4°C with a mixture of primary antibodies, and a 3-h incubation at room temperature with secondary antibodies. Primary antibodies were as follows:

mouse monoclonal anti-Ki67 (1:100; Novocastra Laboratories, Balliol Business Park West, UK), mouse monoclonal anti-p27^{Kip1} (1:100; BD Biosciences), mouse monoclonal anti-NeuN (1:300; Chemicon, Temecula, CA), rabbit polyclonal anti-JSAP1 (1 µg/ml; Miura et al., 2006), mouse monoclonal anti-JNK (1:100; BD Biosciences), mouse monoclonal anti-phospho-JNK (1:100; Santa Cruz Biotechnology, Santa Cruz), rabbit polyclonal anti-phospho-JNK (1:100; Cell Signaling Technology, Beverly, MA), and mouse monoclonal anti-calbindin D28k (calbindin) (1:1000, Sigma) antibodies. Secondary antibodies were goat Alexa fluor 488-conjugated anti-mouse IgG and goat Alexa fluor 568-conjugated anti-rabbit IgG antibodies (both diluted to 1:1000; Invitrogen, Carlsbad, CA). TOTO-3 (Invitrogen) was used as a nuclear stain. Images were captured with a confocal laser scanning microscope (LSM510; Zeiss, Oberkochen, Germany).

Cell cultures

HEK293T cells were cultured as described previously (Bayarsaikhan et al., 2007). Cerebellar GCPs were prepared according to established protocols (Hatten et al., 1998) with minor modifications. Briefly, the cerebellum of P4 mice was removed, and the pia mater peeled off. Tissues were trypsinized with 0.25% trypsin and 0.1% DNase I in Earle's balanced salt solution (EBSS), and then triturated gently with a fire-polished Pasteur pipette to obtain a suspension of single cells. The cells were passed through a 0.22-µm filter, centrifuged, and resuspended in PBS containing 2 mM EDTA. To purify the GCPs, the cells were loaded onto a step gradient of 35% and 60% Percoll (GE Healthcare, Buckinghamshire, UK), and centrifuged at 2000 x g for 20 min at room temperature. The cells at the 35/60% interface were collected, washed in PBS, and resuspended in DMEM/F-12 (Invitrogen) containing 2% BSA. The GCPs were purified further by discarding cells that adhered to tissue culture dishes coated with 50 µg/ml poly-L-lysine (PLL; Sigma) during a 30-min incubation. The non-adhering cells were

collected and resuspended in DMEM/F-12 containing 2% BSA, 5 µg/ml insulin (Sigma), 100 µg/ml apotransferrin (Invitrogen), 40 ng/ml sodium selenite (Sigma), 62 ng/ml progesterone (Sigma), 16 µg/ml putrescine (Sigma), 2.5 mM L-glutamine (Invitrogen), and 25 mM KCl, at a density of 4×10^6 cells/ml. The purified cells were plated onto eight-well Lab-Tec chamber slides (8×10^5 cells/well; Permanox slide; Nunc Inc., Naperville, IL) coated with 50 µg/ml PLL, and cultured at 37°C in a 5% CO₂ atmosphere. For some experiments, Shh (3 µg/ml; R&D Systems, Minneapolis, MN) and/or recombinant lentivirus [1×10^7 transducing units (TU)/well] was added to the culture medium 2 h after plating.

Immunocytochemistry

Cultured GCPs were fixed with 4% PFA/PBS for 30 min at 4°C. Immunocytochemistry was performed by standard protocols using blocking solution (2% BSA, 2% goat serum, 0.4% Triton X-100 in PBS), primary antibodies, and secondary antibodies. Primary antibodies to Ki67, p27^{Kip1}, NeuN, and JSAP1 were as described above, and the others were as follows: rabbit polyclonal anti-GFP (1:300; Chemicon); rat polyclonal anti-HA (1:200; Roche, Welwyn Garden City, UK). Secondary antibodies to mouse and rabbit IgG were as described above, and the other was goat Alexa fluor 488-conjugated anti-rat IgG antibody (1:1000; Invitrogen). Nuclei were stained with 2 µg/ml Hoechst 33258 (Invitrogen). For double staining using anti-BrdU and anti-GFP or anti-HA antibodies, cultured GCPs were first stained with the anti-GFP or anti-HA antibodies, then refixed and treated with 2 N HCl in double-distilled water for 20 min at 37°C, and then stained with the anti-BrdU antibody. Photographs were taken with an IX71 inverted microscope (Olympus, Tokyo, Japan) attached to a DP50 digital CCD camera (Olympus).

Plasmids

The mammalian expression plasmid for Flag-tagged JSAP1, pcDNA3-Flag-JSAP1, was described previously (Bayarsaikhan et al., 2006). To generate an expression plasmid for a short hairpin RNA (shRNA) against JSAP1, the following annealed oligonucleotides were inserted into pSUPER (OligoEngine; Seattle, WA): forward, 5'-GATCCCCGTGTCACAAAGAACAACAATTCAAGAGATTGTTGTTCTTTGTGACACTTTTTGGAAA-3'; reverse, 5'-AGCTTTTCCAAAAAGTGTCACAAAGAACAACAATCTCTTGAATTGTTGTTCTTTGTGACACGGG-3'. The underlined letters represent mouse JSAP1 sequences (GenBank accession number AB043124). As a control, an shRNA against β -galactosidase was made, in which the following annealed oligonucleotides were inserted into pSUPER (OligoEngine; Seattle, WA): forward, 5'-GATCCCCGTGAAAACATAAAACACTGGTTCAGAGACCAGTGTTTTAGTTTTCACTTTTTGGAAA-3'; reverse, 5'-AGCTTTTCCAAAAAGTGAAAACATAAAACACTGGTCTCTTGAACCACTGTTTTAGTTTTCACGGG-3'. The underlined letters represent *Escherichia coli* β -galactosidase sequences (GenBank accession number V00296). The H1 promoter-JSAP1/ β -galactosidase-shRNA cassettes in the resultant plasmids were inserted into the lentiviral vector pLVTH (Addgene plasmid 12262; Addgene, Cambridge, MA), to generate pLVTH-JSAP1-shRNA and pLVTH- β -galactosidase-shRNA, respectively. In pLVTH, GFP is encoded under the control of the human elongation factor 1 alpha promoter, as a marker. The envelope plasmid pMD2.G, encoding vesicular stomatitis virus glycoprotein (VSV-G) (Addgene plasmid 12259), and the packaging plasmid psPAX2 (Addgene plasmid 12260) were provided by Addgene. The murine stem cell virus (MSCV) promoter in the lentiviral vector pCL20c MSCV-GFP (Hanawa et al., 2004) was replaced by the cytomegalovirus (CMV) immediate-early promoter, to generate pCL20c CMV-GFP. Expression plasmids for HA-tagged wild-type JSAP1, HA-tagged mutant

JSAP1 lacking the JBD (Sato et al., 2004), Flag-tagged GFP, and Flag-tagged JBD (amino acid residues 201-220 of mouse JSAP1) fused to GFP were generated by replacing the GFP coding sequence in pCL20c CMV-GFP with the coding sequence for the respective construct. The resultant plasmids were designated pCL20c CMV-HA-JSAP1(WT), pCL20c CMV-HA-JSAP1(Δ JBD), pCL20c CMV Flag-GFP, and pCL20c CMV-Flag-GFP-JBD, respectively. The helper plasmids pCAGkGP1R, pCAG4RTR2, and pCAG-VSV-G as well as pCL20c MSCV-GFP were kindly provided by Dr. Arthur W. Nienhuis (St. Jude Children's Research Hospital).

Viral vector preparation

VSV-G-pseudotyped human immunodeficiency virus vectors were used in this study. The pCL20c series of viral vectors was produced by the cotransfection of HEK293T cells with a mixture of four plasmids, pCAGkGP1R, pCAG4RTR2, pCAG-VSV-G, and pCL20c, using a calcium phosphate precipitation method as described previously (Torashima et al., 2006a, 2006b). The pLVTH series of viral vectors was also produced, by the transient transfection of HEK293T cells plated in 100-mm dishes, with similar procedures: the plasmids used for each transfection were 15 μ g pLVTH or pLVTH-JSAP1-shRNA, 11.25 μ g psPAX2, and 3.75 μ g of pMD2.G. The medium, containing the viral particles, was harvested 64 h after the transfection. The medium samples were filtered through 0.45- μ m membranes, centrifuged at 25,000 rpm for 90 min, resuspended in PBS (pH 7.4), and stored as frozen aliquots at -80°C until use. The titers (TU/ml) of virus stocks were measured as described previously (Torashima et al., 2006a).

Western blotting

HEK293T cells were transfected with expression plasmids using a calcium phosphate

precipitation method. Forty-eight hours after the transfection, the cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P40, 0.1% sodium deoxycholate) containing Protease Inhibitor Cocktail (Sigma). Western blot analysis was carried out as described previously (Sato et al., 2004), using mouse monoclonal anti-Flag M2 (1:5000; Sigma), mouse monoclonal anti-HA (1:2000; Invivogen), mouse monoclonal anti- α -tubulin (1:5000; Sigma), rabbit polyclonal anti-c-Jun (1:1000; Cell Signaling Technology), and rabbit polyclonal anti-phospho-c-Jun (1:1000; Cell Signaling Technology) antibodies. Protein bands were visualized by the ECL Plus chemiluminescence system (GE Healthcare).

RT-PCR

GCPs were prepared as described above. To examine the role of JSAP1 during GCP differentiation, cells (1×10^6 cells) were transfected with 500 ng pCL20c CMV-GFP alone, or with 500 ng of either pCL20c CMV-HA-JSAP1(WT) or pCL20c CMV-HA-JSAP1(Δ JBD) using the Mouse Neuron Nucleofector Kit (Amaxa Biosystems, Cologne, Germany). After 48 h of culture, GFP-positive GCPs were sorted by using a FACS (FACS Aria; BD Biosciences). For the analysis of the expression of *Jnk* genes, GCPs prepared as described above were used as RNA sources. Total RNAs were extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and the RNA samples were treated with RNase-free DNase I (Promega, Madison, WI) to eliminate genomic DNA contamination. Real time RT-PCR was performed as described previously (Sato et al., 2005). The expression level of each mRNA was normalized with respect to that of *Gapdh* mRNA. The following primers were used: *Jnk1*, 5'-AAGCCTATAGGCTCAGGAGCT-3' and 5'-AGAACTAGTTCTCGGTAGGCG-3'; *Jnk2*, 5'-CCTTCTCTACCAGATGCTCTG-3' and 5'-AAGTTGGTACAGGCTGTTCGC-3'; *Jnk3*,

5'-GTGGCCATTAAGAAGCTCAGC-3' and 5'-ACTCCTCCAGTGTTTTCTGGG-3';
Neurod1, 5'-TCAAAGCCAACGATCGCGAGC-3' and
5'-GTAGTTGTAGGCGAAGCGCAG-3'; *Nfia*, 5'-ATGAGTCCAGGAGCAATGAGG-3'
and 5'-CGCCCTTGGCCTGTGTAAAAT-3'; *Pax6*,
5'-TGCTTGGGAAATCCGAGACAG-3' and 5'-GCCCATCTGTTGCTTTTCGCT-3';
Gapdh, 5'-ATGTGTCCGTCGTGGATCTGA-3' and
5'-TTGCTGTTGAAGTCGCAGGAG-3'.

Luciferase assay

GCPs were prepared as described above. Cells (1×10^6 cells) were cotransfected with 100 ng pFR-Luc, 100 ng pFA-cJun, 40 ng pRL-TK Luc, and 0, 100, 200, or 500 ng of either pCL20c CMV-HA-JSAP1(WT) or pCL20c CMV-HA-JSAP1(Δ JBD) using the Mouse Neuron Nucleofector Kit (Amaxa Biosystems). pCL20c CMV-Flag-GFP was used to normalize the total quantity of transfected DNA (1 μ g/transfection). The pFR-Luc vector contains a synthetic promoter with five tandem repeats of the yeast GAL4 binding sites and the firefly luciferase gene. The pFA-cJun vector expressing the fusion *trans*-activator protein consists of the activation domain of *c-Jun* fused with the DNA-binding domain of the yeast GAL4. The pRL-TK Luc plasmid contains the herpes simplex virus thymidine kinase promoter to provide moderate levels of *Renilla* luciferase expression. After 48 h of culture, the cells were lysed and assayed by using the Dual Luciferase Assay system (Promega). Firefly luciferase activity in the lysates was normalized using the corresponding internal *Renilla* luciferase activity, and then divided by the value for control cultures to calculate the fold change in luciferase activity.

Statistical analysis

Results are represented as the mean \pm SEM from the number of experiments indicated in the figure legends. Statistical differences were analyzed using the two-tailed unpaired Student's *t*-test (Figs. 3, 4, 5, and 9), or a one-way ANOVA followed by the Tukey's multiple comparison *pos hoc* test (Figs. 6, 7, and 8). Values of $p < 0.05$ were considered to be statistically significant.

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Acknowledgements

We thank Dr. T. Yoshihara (Kanazawa University) for helpful discussion. This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Figure legends

Figure 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.

Figure 2. Expression of JSAP1 and JNK in developing mouse cerebellum. **A-P**, Sagittal sections from P6 mouse cerebella double-labeled with anti-JSAP1 (red) and (in green) anti-JNK (**A-H**) or anti-phosphorylated, activated JNK (P-JNK) (**I-P**). Lower panels (**E-H** and **M-P**) show high-magnification views of upper panels (**A-D** and **I-L**); the area shown is indicated in **C** for **A-D** and in **K** for **I-L**. Immunosignals for JNK largely overlapped with those for JSAP1. The expression profile of P-JNK was also similar to that of JSAP1, with small differences. There was little P-JNK immunoreactivity in the outer EGL. In the inner EGL, where P-JNK levels were high, the P-JNK-positive cells were also JSAP1-positive. However, just superficial to this region, there was a thin stripe of cells that were positive for JSAP1 that expressed only low levels of P-JNK. **D, H, L, and P**, Nuclei stained with TOTO-3 (blue). outer, outer EGL; inner, inner EGL. Scale bars: 20 μm in **A-D** and **I-L**; 10 μm in **E-H** and **M-P**. Images were captured with a confocal

microscope.

Figure 3. Expression of Ki67 and p27^{Kip1} in cultured cerebellar GCPs. GCPs prepared from P4 mice were cultured with or without Shh for 24 h or 72 h, fixed, and stained with antibodies against Ki67 and p27^{Kip1}. Percentage of Ki67 and p27^{Kip1}-expressing cells (means \pm SEM from 3 experiments, * $p < 0.01$, Student's t -test). A minimum of 1,000 cells was counted per experiment. GCPs can differentiate in this culture system, and therefore the proportions of Ki67- and p27^{Kip1}-positive cells decreased and increased, respectively, with time. The mitogen Shh enhanced the proportion of proliferating Ki67-positive cells, resulting in a decreased population of cells expressing p27^{Kip1}, a negative regulator of the cell cycle in GCPs.

Figure 4. Knockdown of JSAP1 expression enhances the proliferation of cultured cerebellar GCPs. **A**, Representative Western blot (WB) shows the effective silencing of JSAP1 expression by the shRNA for JSAP1 (JSAP1-shRNA), evaluated by transient coexpression of the shRNA with Flag-tagged JSAP1 in HEK293T cells. A vector expressing β -galactosidase shRNA (control-shRNA) was used as the negative control. Expression of α -tubulin is shown as the loading control. **B**, Double immunofluorescence shows a much lower expression of JSAP1 in cultured P4 GCPs infected with a lentivirus containing the JSAP1-shRNA than in those infected with lentivirus containing the control shRNA. The cells were stained with antibodies to GFP (green) and JSAP1 (red) 72 h after infection. **C**, Double immunofluorescence of cultured P4 GCPs infected with lentivirus expressing GFP plus either control-shRNA (control-shRNA) or JSAP1-shRNA (JSAP1-shRNA), 72 h after infection. Before fixation, the cells were labeled with BrdU for 12 h. They were then stained with antibodies to GFP (green) and BrdU (red). Images were captured with an inverted microscope (**B**, **C**). Scale bars, 20 μ m in **B** and **C**. **D**, The percentage of GFP-positive cells that were BrdU-positive in cultures infected with the

control lentivirus or lentivirus containing the JSAP1-shRNA (means \pm SEM from 3 experiments, $*p < 0.005$, Student's *t*-test).

Figure 5. Inhibition of JNK activity enhances the proliferation of cultured cerebellar GCPs. **A**, Representative Western blot (WB) showing that a fusion protein of the JSAP1 JNK-binding domain (JBD) with Flag-tagged GFP (Flag-GFP-JBD) acts as a JNK inhibitor. HEK293T cells were transiently transfected with expression vectors for Flag-GFP or Flag-GFP-JBD and stimulated with UV (40 J/m^2) or left unstimulated. Total cell lysates were prepared and used for WB analysis with antibodies to phospho-c-Jun (P-c-Jun), c-Jun, Flag-tag, and α -tubulin. **B**, Double immunofluorescence of P4 GCPs expressing lentivirus-mediated Flag-GFP or Flag-GFP-JBD, 72 h after infection, stained for GFP (green) and BrdU (red). Before fixation, the cells were labeled with BrdU for 12 h. Images were captured with an inverted microscope. Scale bars, $20 \mu\text{m}$. **C**, Percentage of GFP-positive cells that were also positive for BrdU in cultures infected with lentivirus for Flag-GFP or Flag-GFP-JBD (means \pm SEM from 3 experiments, $*p < 0.01$, Student's *t*-test).

Figure 6. JSAP1 promotes $p27^{\text{Kip1}}$ expression in cultured cerebellar GCPs and suppresses proliferation. **A**, Western blot shows similar levels of protein expression in HEK293T cells transfected with HA-tagged wild-type JSAP1 (HA-JSAP1(WT)) or its mutant lacking the JBD (HA-JSAP1(Δ JBD)). **B**, **C**, P4 GCPs cultured for 72 h with Shh ($3 \mu\text{g/ml}$) and infected with lentivirus expressing GFP (control), HA-JSAP1(WT), or HA-JSAP1(Δ JBD), and stained for GFP or HA and $p27^{\text{Kip1}}$ or BrdU. Percentage of GFP- or HA-positive cells that were $p27^{\text{Kip1}}$ -positive (**B**) or BrdU-positive (**C**) (means \pm SEM from 3 experiments, $*p < 0.01$, one-way ANOVA). HA-JSAP1(WT) lentivirus-infected cells showed a significantly greater percentage of $p27^{\text{Kip1}}$ -positive cells than in cultures infected with the control lentivirus, in the absence and presence of Shh. Much less enhancement of the

proportion of p27^{Kip1}-positive cells ($p < 0.01$ compared with HA-JSAP1(WT)) was seen in cultures of HA-JSAP1(Δ JBD) lentivirus-infected cells, and concomitantly, the proportion of BrdU-positive proliferating GCPs increased.

Figure 7. JSAP1 promotes the differentiation of cultured cerebellar GCPs. P4 GCPs cultured for 72 h in the presence of Shh (3 μ g/ml) and infected with lentiviruses for GFP (control), HA-JSAP1(WT), or HA-JSAP1(Δ JBD), were evaluated by double immunofluorescence staining for GFP or HA, and NeuN, a neural differentiation marker. Percentage of GFP- or HA-positive cells that were positive for NeuN (means \pm SEM from 3 experiments, $*p < 0.01$, one-way ANOVA). Although HA-JSAP1(WT) induced a significant increase in GCP differentiation, HA-JSAP1(Δ JBD) had a significantly less strong effect on differentiation ($p < 0.01$) compared with HA-JSAP1(WT) in cultures of lentivirus-infected cells in both the absence and presence of Shh.

Figure 8. JSAP1 enhances the expression of markers for postmitotic neuroblasts in cultured cerebellar GCPs. GCPs prepared from P4 mice were transfected with the expression plasmid for GFP alone (control), or with HA-JSAP1(WT) or HA-JSAP1(Δ JBD), and GFP-positive cells were purified by FACS. Forty-eight hours after transfection, total RNAs were prepared from the cells, and subjected to real-time RT-PCR using the respective gene-specific primers as indicated. The expression levels of the mRNAs were normalized to that of *Gapdh* mRNA, and the quantity of each mRNA was determined as its ratio to the level in control cells. Values are given as means \pm SEM from 3 experiments, $*p < 0.05$, one-way ANOVA. The expression levels of all the markers were significantly higher in cells expressing HA-JSAP1(WT) compared with those expressing HA-JSAP1(Δ JBD).

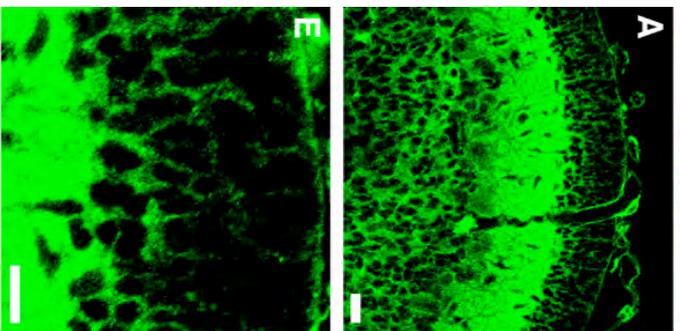
Figure 9. JSAP1 overexpression in cultured cerebellar GCPs causes increased JNK

activity. GCPs prepared from P4 mice were cotransfected with the indicated amount of the expression plasmids for HA-JSAP1(WT) or HA-JSAP1(Δ JBD) together with firefly and *Renilla* luciferase reporter plasmids. Forty-eight hours after transfection, the cells were assayed for luciferase activity. The forced expression of JSAP1(WT) increased the JNK activity in a dose-dependent manner, and this effect was significantly suppressed when JSAP1(Δ JBD) was used instead of JSAP1(WT) (means \pm SEM from 3 experiments, $*p < 0.005$, Student's *t*-test).

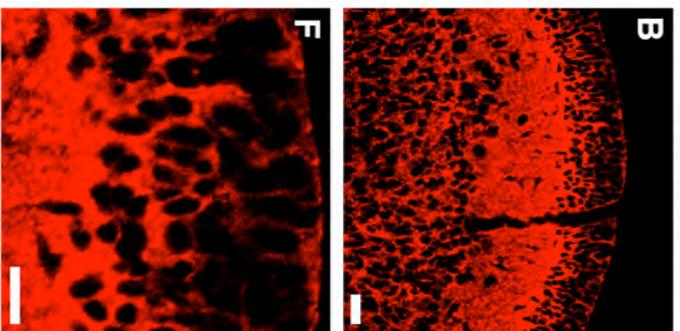
Supplementary Figure 1. Expression of JSAP1, P-JNK, and calbindin in developing mouse cerebellum. Sagittal sections from P6 mouse cerebella double-labeled with anti-calbindin (green) and (in red) anti-JSAP1 (**A, B**) or anti-P-JNK (**D, E**). **B** and **E** show high-magnification views of the areas indicated in **A** and **D**, respectively. **C** and **F** show nuclei stained with TOTO-3 (blue). Immunosignals for calbindin, a Purkinje cell marker, were detected beneath the EGL, but not in it, and some calbindin-positive cells were also positive for JSAP1 and P-JNK. calb, calbindin; outer, outer EGL; inner, inner EGL. Scale bars: 20 μ m in **A** and **D**; 10 μ m in **B, C, E**, and **F**. Images were captured with a confocal microscope.

Supplementary Figure 2. Expression of *Jnk* genes in cerebellar GCPs of P4 mice. Total RNAs were prepared from P4 GCPs, and real-time PCR analysis was performed using the respective gene-specific primers as indicated. The expression levels of the mRNAs were normalized to that of *Gapdh* mRNA, and the quantity of each mRNA was determined as its ratio to the level of *Jnk1* mRNA. Values are given as means \pm SEM from 3 experiments. The expression of *Jnk3* was highest among the *Jnk* genes in the GCPs.

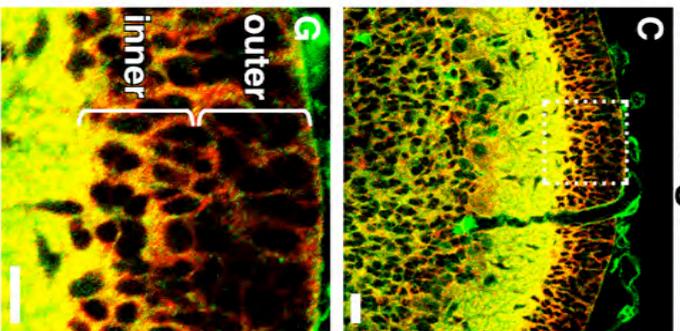
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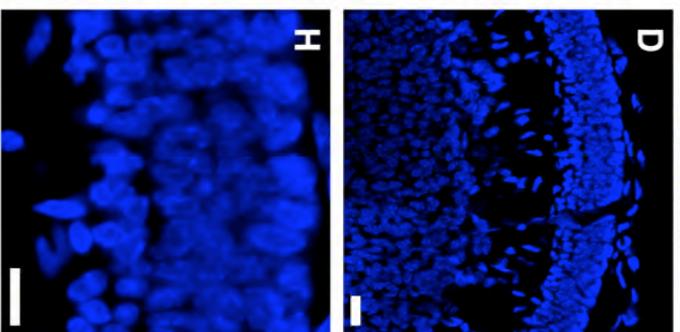
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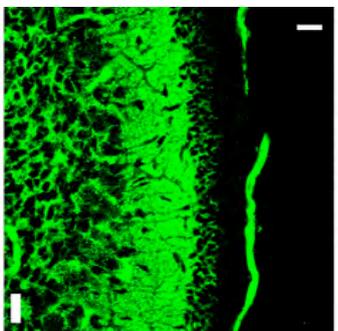
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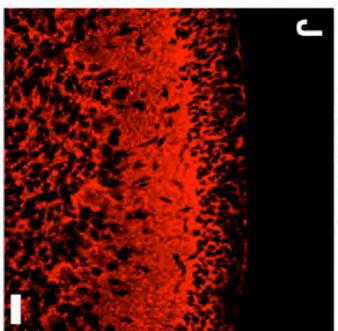
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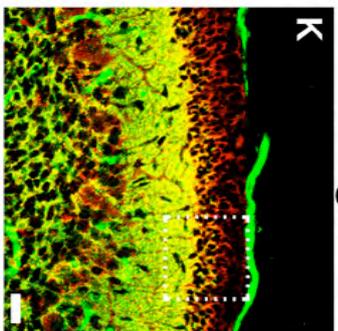
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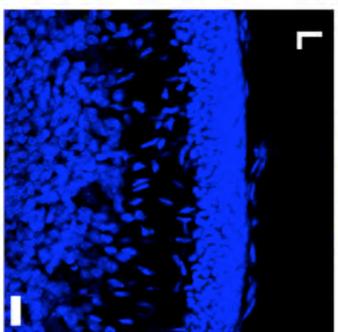
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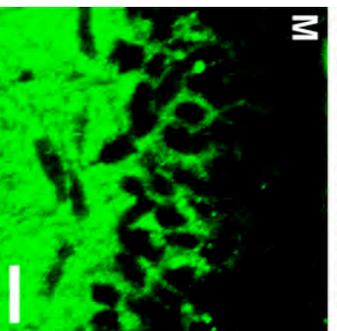
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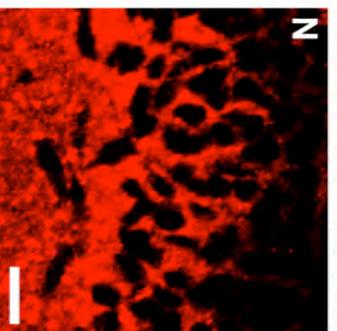
TOTO-3



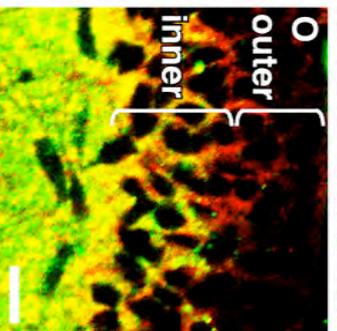
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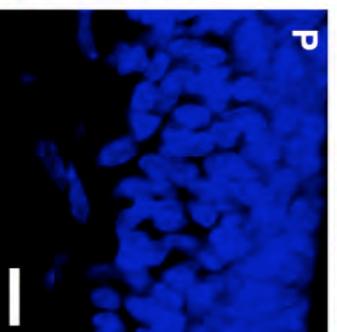
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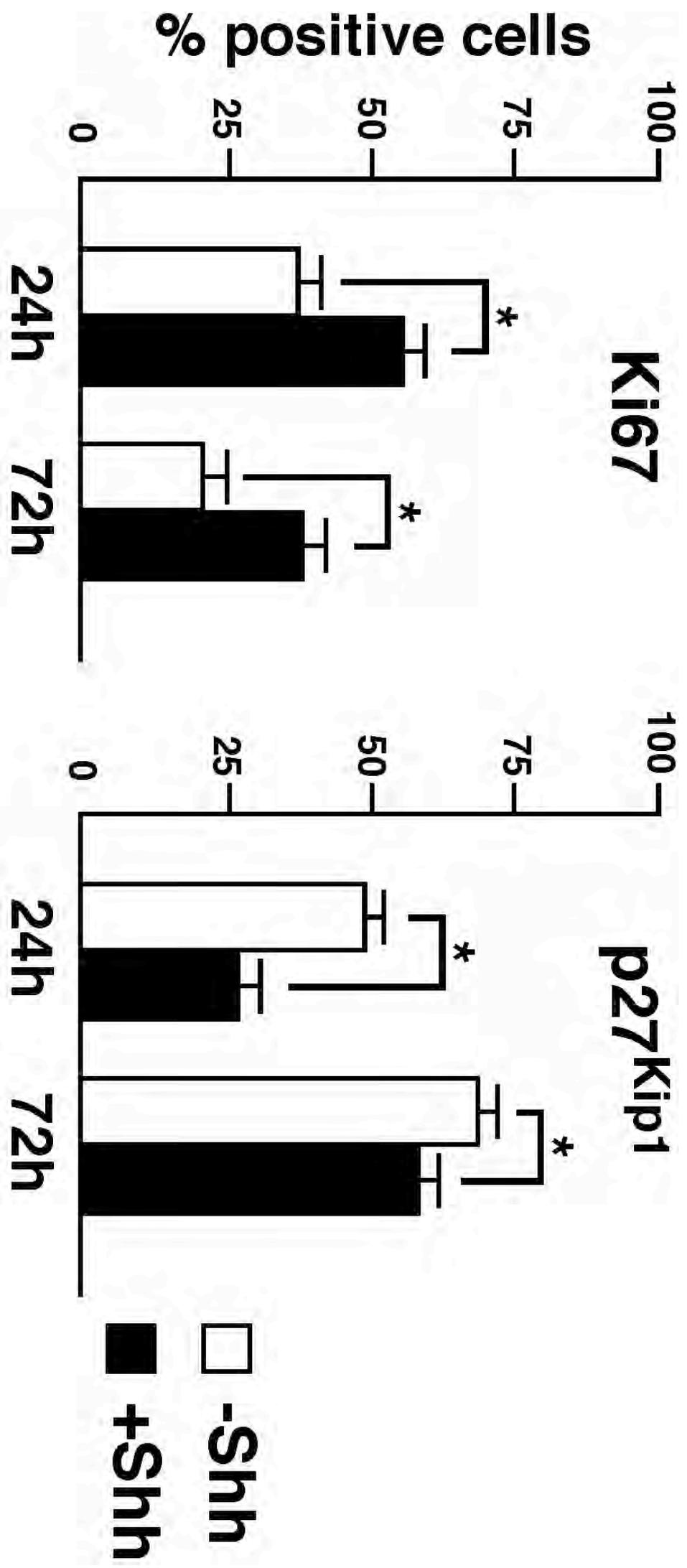


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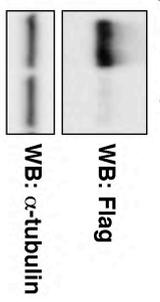
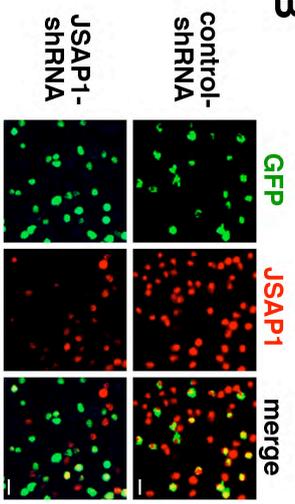
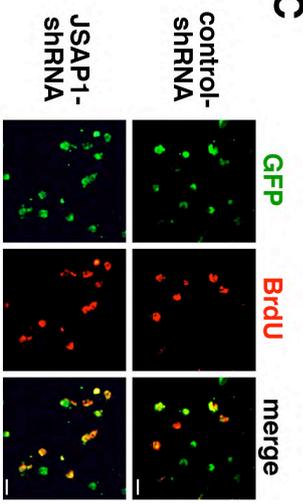
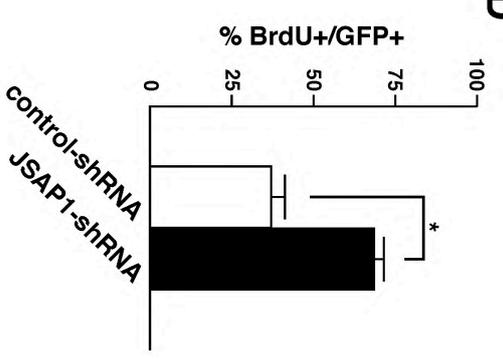
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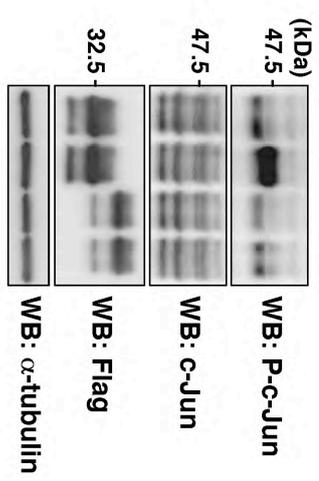
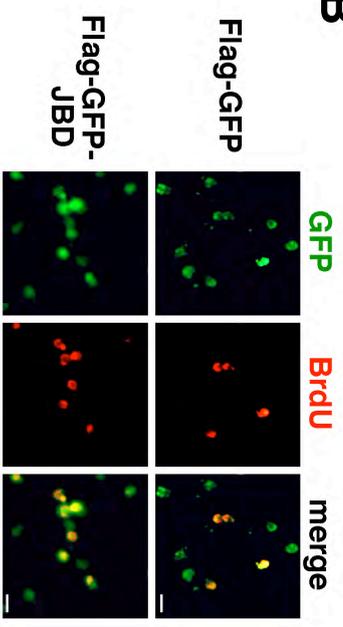
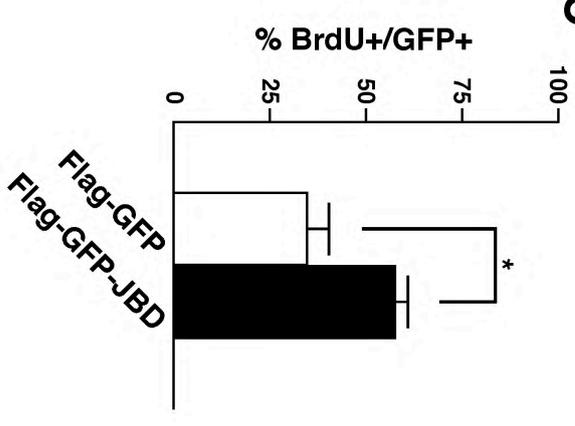
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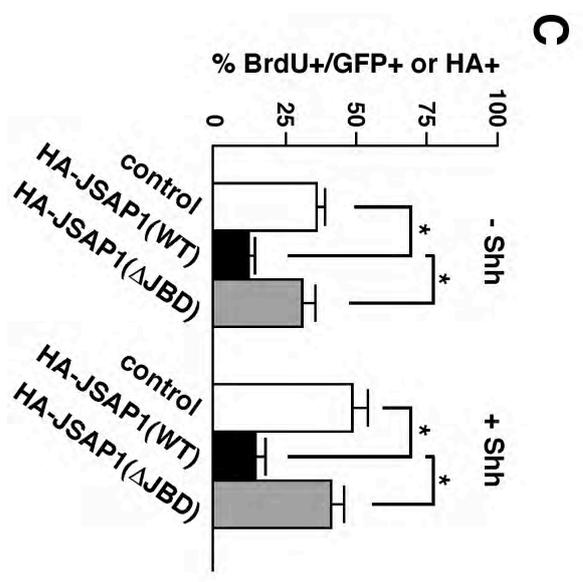
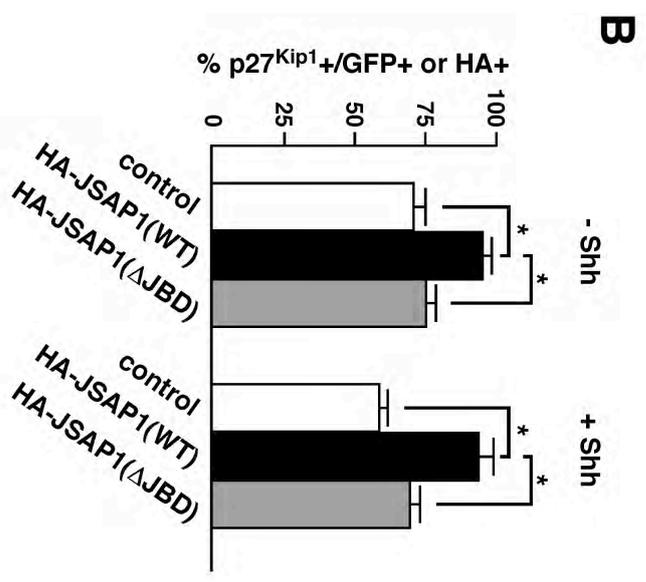
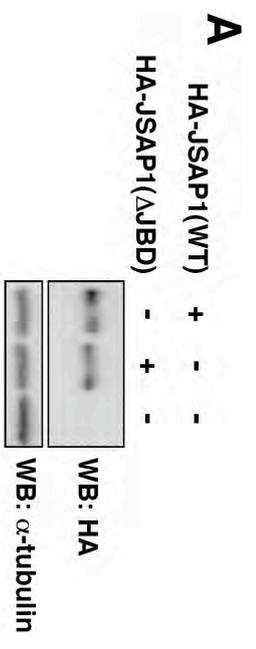
Flag-JSAP1	+	+
control-shRNA	+	-
JSAP1-shRNA	-	+

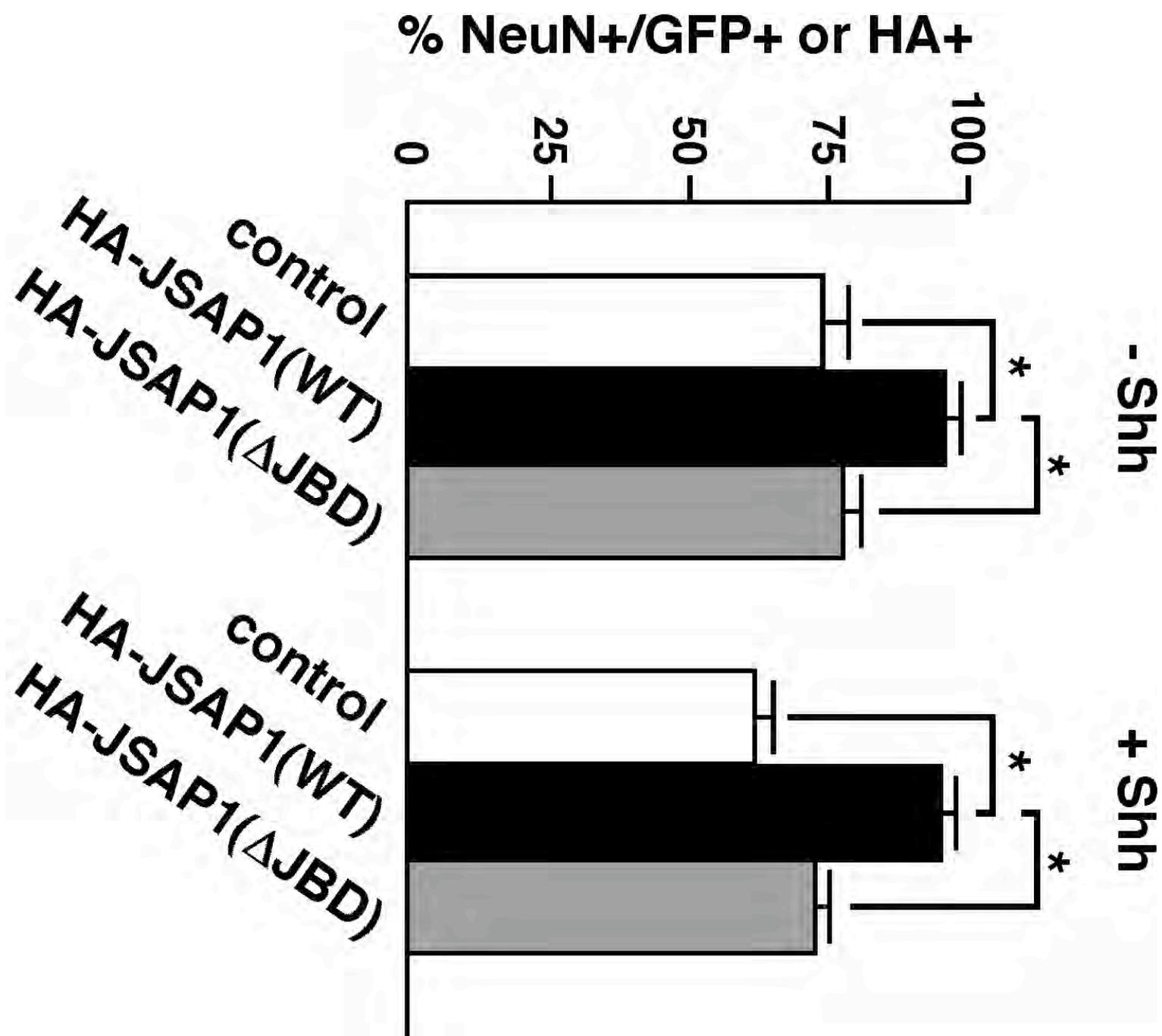
**B****C****D**

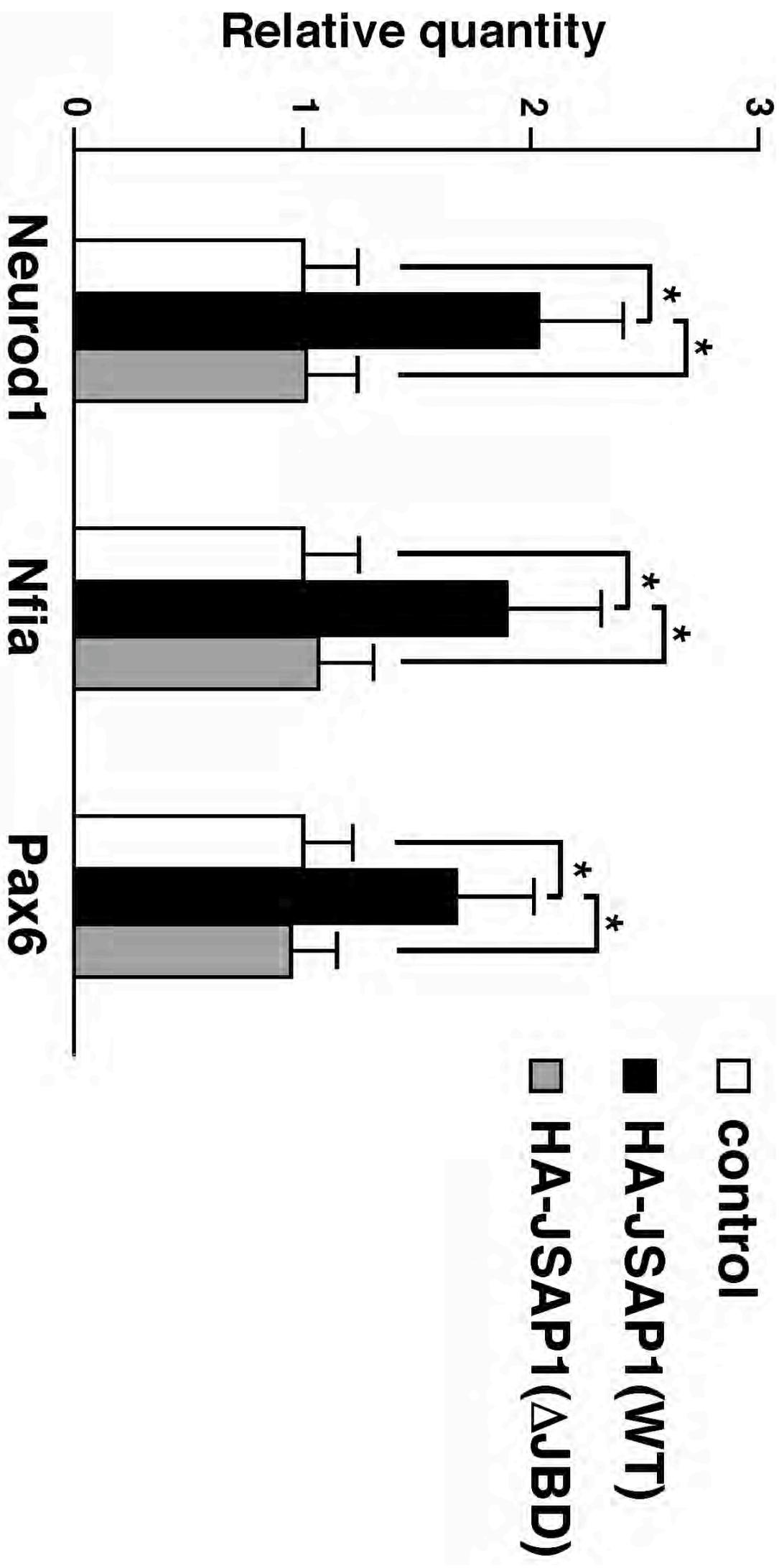
A

UV	-	+	-	+
Flag-GFP	+	+	-	-
Flag-GFP-JBD	-	-	+	+

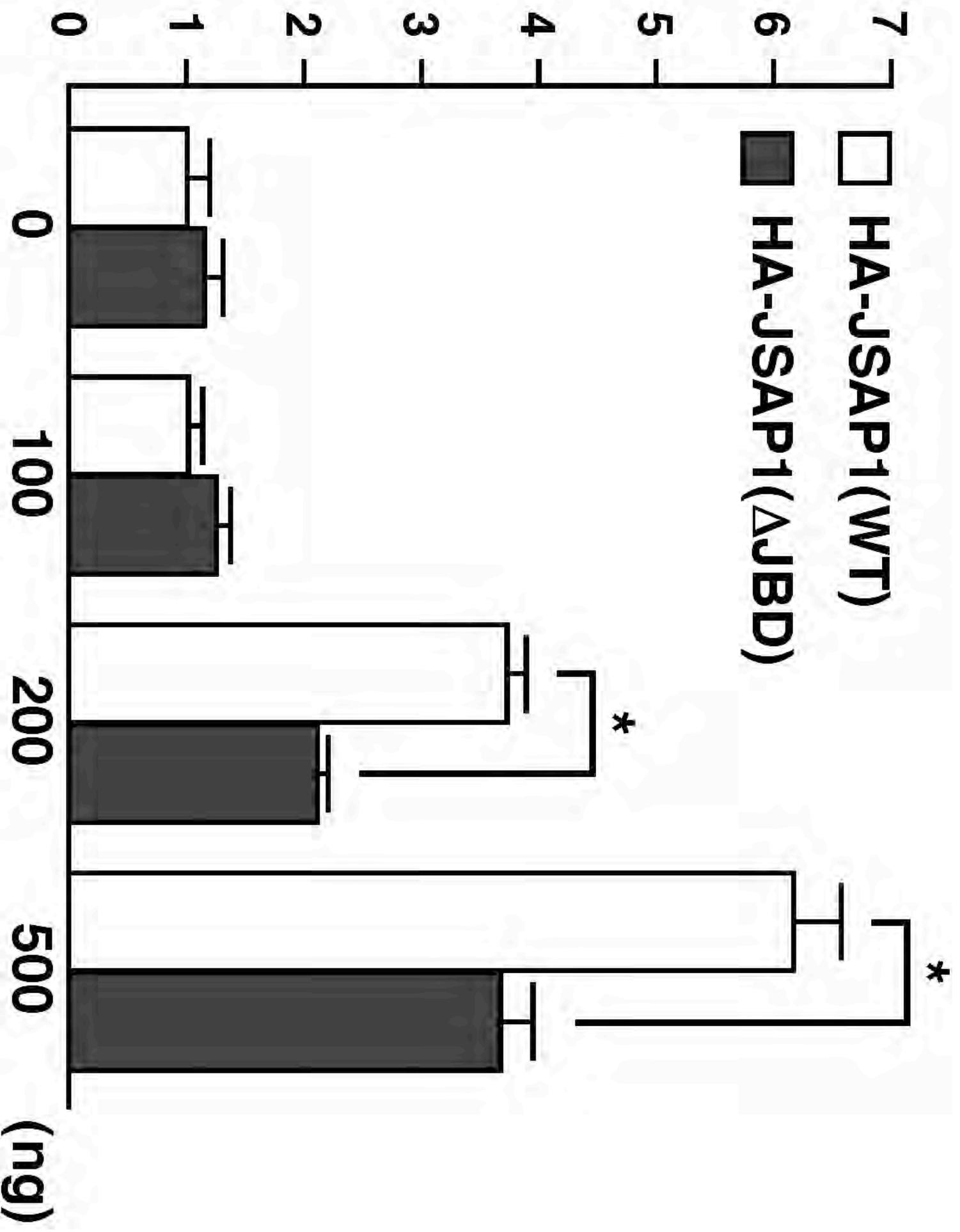
**B****C**





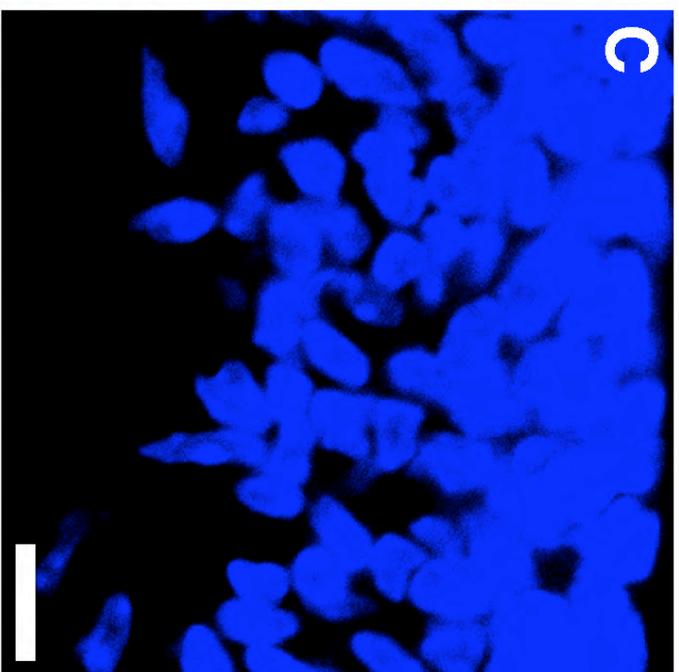
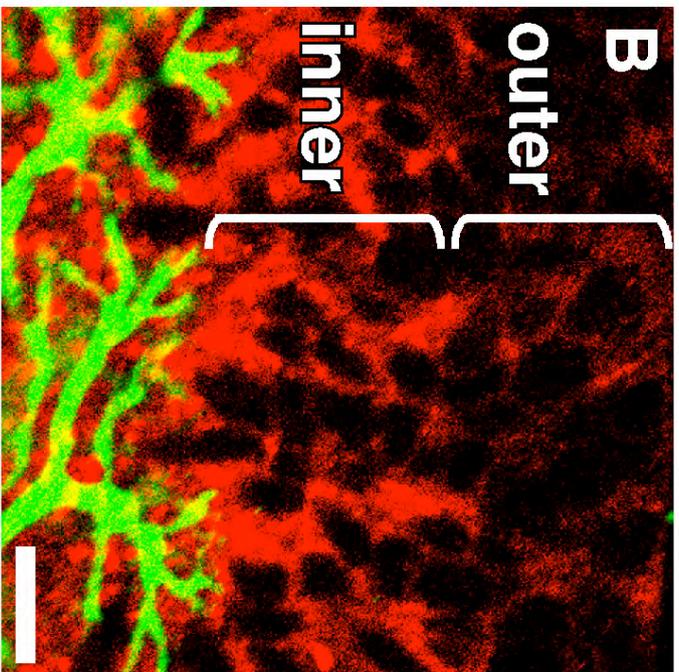
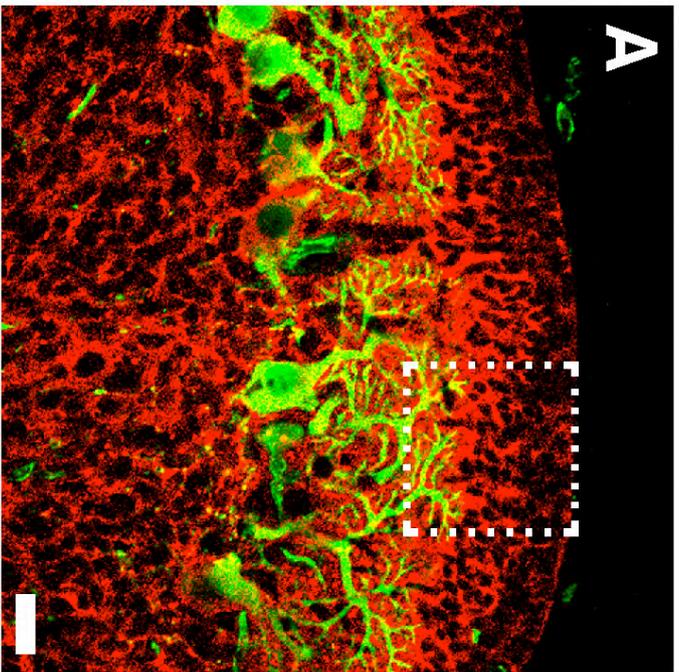


Luciferase activity (fold change)

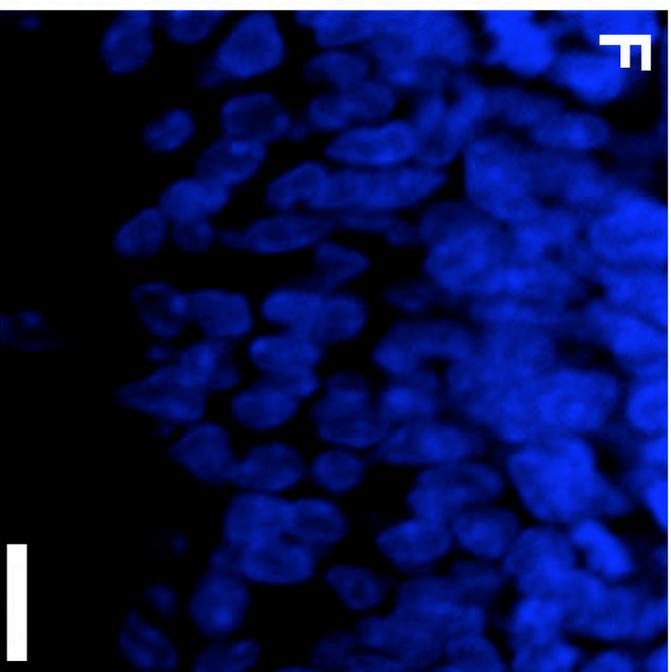
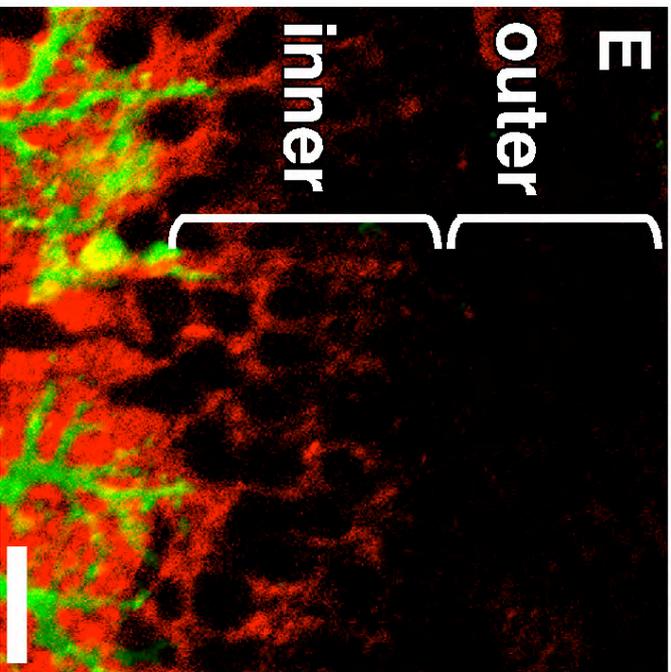
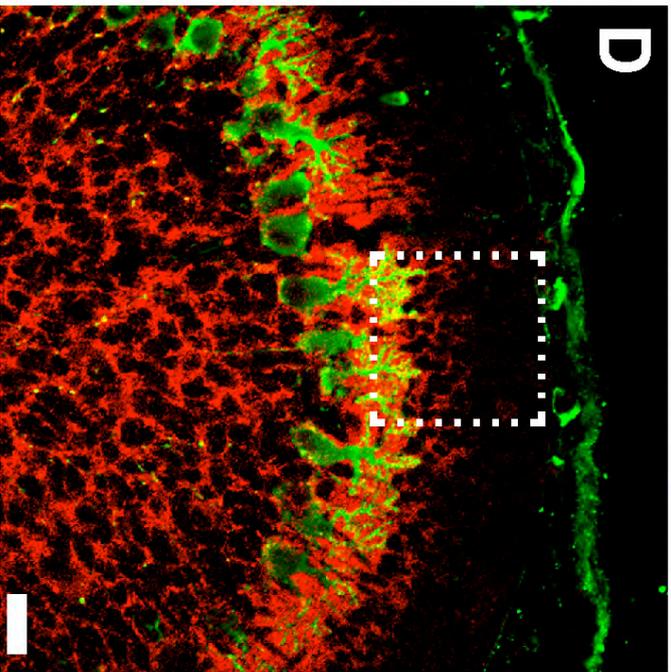


TOTO-3

calb/JSAP1



calb/P-JNK



Relative quantity

