Antiapoptotic protein Lifeguard is required for survival and maintenance of Purkinje and granular cells

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Lifeguard (LFG) is an inhibitor of Fas-mediated cell death and is highly expressed in the cerebellum. We investigated the biological role of LFG in the cerebellum in vivo, using mice with reduced LFG expression generated by shRNA lentiviral transgenesis (shLFG mice) as well as LFG null mice. We found that LFG plays a role in cerebellar development by affecting cerebellar size, internal granular layer (IGL) thickness, and Purkinje cell (PC) development. All these features are more severe in early developmental stages and show substantial recovery overtime, providing a remarkable example of cerebellar plasticity. In adult mice, LFG plays a role in PC maintenance shown by reduced cellular density and abnormal morphology with increased active caspase 8 and caspase 3 immunostaining in shLFG and knockout (KO) PCs. We studied the mechanism of action of LFG as an inhibitor of the Fas pathway and provided evidence of the neuroprotective role of LFG in cerebellar granule neurons (CGNs) and PCs in an organotypic cerebellar culture system. Biochemical analysis of the Fas pathway revealed that LFG inhibits Fas-mediated cell death by interfering with caspase 8 activation. This result is supported by the increased number of active caspase 8-positive PCs in adult mice lacking LFG. These data demonstrate that LFG is required for proper development and survival of granular and Purkinje cells and suggest LFG may play a role in cerebellar disorders.

The cerebellum is a laminated brain structure that comprises three major groups of cells: Purkinje cells (PCs), cerebellar granule neurons (CGNs), and deep cerebellar neurons. PCs are inhibitory GABAergic neurons that originate in the ventricular zone of the cerebellar primordium and migrate to the cerebellar plate aggregating in a 6- to 10-cell-thick layer before evolving into a monolayer located adjacent to the internal granular layer (IGL; refs. 1 and 2). CGN progenitors are generated in the rhombic lip and migrate to form the external granule layer (EGL) where their proliferation will be driven by PC secretion of sonic hedgehog (3). In turn cells in the EGL will promote PC migration by secretion of Reelin (4). CGNs will then migrate across the PC layer, guided by the radial glia, to form the IGL (5, 6).

Many different mechanisms trigger neuronal apoptosis. However, the mechanisms involved in programmed cell death (PCD) in the central nervous system (CNS) are not fully understood. One of these apoptotic mechanisms involves the Fas receptor (FasR), one of the most investigated death receptor family members. Although Fas-mediated cell death is primarily found and characterized in the immune system, FasR was also demonstrated to influence neuronal survival following trophic factor deprivation (7). Furthermore, CGNs from mice with mutated Fas Ligand (FasL) are more resistant to trophic factor deprivation in culture (8). Other components of the Fas apoptotic pathway, such as caspase 3, have been implicated in PCD of postmitotic postmigratory neurons during the development of the cerebellar cortex (9).

Lifeguard (LFG) is an antiapoptotic protein that antagonizes the Fas pathway (10). Its transcripts and protein are strongly upregulated during postnatal brain development (11, 12) and are highly expressed in PCs and CGNs, suggesting a role in development and/or survival of these neurons. This hypothesis was partially investigated in two in vitro studies that found that PI3K-dependent expression of LFG conferred resistance to Fas-mediated apoptosis in CGNs, thereby suggesting that LFG regulation can play a role in the survival of these cerebellar neurons (13, 14). However, the role of LFG in development and survival of PCs remains to be investigated.

We generated mouse models in which LFG levels were severely reduced by shRNA lentiviral transgenesis or completely eliminated in LFG null mice. We demonstrate that LFG plays a role in cerebellar cell development and survival, affecting mainly PCs and CGNs. Furthermore, we show that organotypic cerebellar cultures from LFG KO mice are more sensitive to Fas-mediated cell death. Our data support a role for LFG in neuroprotection of PCs.

Results

LFG Protects Against Fas-Mediated Apoptosis by Interfering with Caspase 8 Activation. HeLa cells are known to be sensitive to Fas-mediated cell death. Expression of LFG in these cells confers them resistance (Fig. S1). To investigate how LFG blocks the Fas apoptotic cascade, we decided to examine the recruitment of the Death-Induced Signaling Complex (DISC) components in HeLa cells and HeLa cells expressing LFG. Therefore, we performed DISC immunoprecipitations (IP) with a Fas activating antibody to see whether FADD and caspase 8 were recruited. The IP-Western blot (WB) showed that, in both HeLa and HeLa-LFG cells treated with Fas antibody, FADD and caspase 8 were successfully recruited, indicating that LFG does not interfere with the recruitment of the DISC components. On the other hand, we observed a marked reduction of caspase 8 cleavage in the Fas-treated HeLa-LFG cells where only 44% of the recruited caspase 8 was cleaved compared with 81% in the HeLa cells (Fig. 1 *A*, lanes 2 and 4, and *B*), suggesting that LFG interferes with caspase 8 activation but not with its recruitment to the DISC.

To study the dynamics of caspase 8 activation in HeLa and HeLa-LFG cells, we performed a caspase 8 activity assay coupled to a cell viability assay. The results showed that HeLa cells have a higher caspase 8 activity, which is followed by a very significant decline in cell viability over time (2.7% viable cells at 24 h), whereas the HeLa-LFG cells have lower caspase 8 activity that translates into a much smaller decline in cell viability (59% viable cells at 24 h; Fig. 1*C*). Our data suggest that the expression of LFG interferes with the activation of caspase 8 allowing the cells to escape apoptosis.

LFG Knockdown and KO Mice. To study the function of LFG in vivo, we used LFG knockdown and KO animals. Mice with reduced LFG expression were generated by Lentiviral-shRNA transgenesis (15). shRNAs against mouse LFG were cloned into the U3 region of a lentiviral vector containing a GFP cassette driven by the mouse PGK promoter. The shRNAs are under the mouse U6 Pol III promoter; therefore, LFG is down-regulated from its

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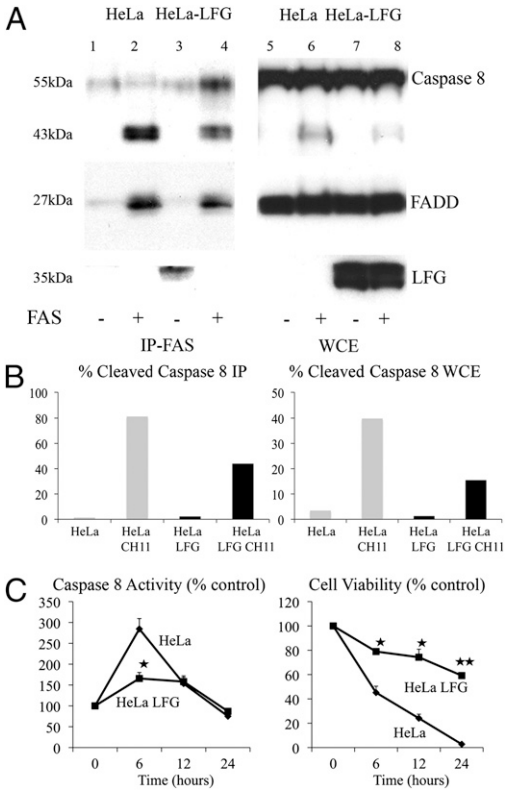

Fig. 1. LFG interferes with caspase 8 activation. (*A*) WB of DISC IP with Fas antibody on protein extracts from untreated and Fas-treated HeLa and HeLa-LFG cells. Immunoprecipitates (IP) and whole cell extracts (WCE) were resolved by SDS/PAGE and immunoblotted with caspase 8, FADD, and FlagM2 (LFG) antibodies. Lanes 2 and 4 show how FADD and caspase 8 are recruited to the DISC regardless of LFG expression, however the caspase 8 panel shows a higher amount of cleaved caspase in wild type HeLa cells compared with HeLa LFG cells. Lanes 3 and 4 of the LFG panel show that LFG immunoprecipitates with Fas only in the untreated condition. (*B*) Histograms of the quantification of the bands of procaspase 8 and its cleaved form. Results are expressed in percentage of cleaved caspase 8. (*C*) Profile of caspase 8 activity and cell viability at the indicated time points after addition of CH11. Results are expressed in percentage of the value of the untreated cells at each time point. $*P \leq 0.05$, $**P \leq 0.01$, ± SEM.

first expression. LFG KO mice were generated by Deltagen. The targeting strategy consisted in the insertion of a LacZ-Neomicin cassette between exons 4 and 5 of LFG's genomic sequence. Validation of the mouse models was carried out by quantitative PCR (QPCR) of cerebellar cDNA (Figs. S2 and S3).

LFG Expression in the Brain. To study the expression pattern of LFG in the mouse brain, we performed in situ hybridization (ISH) with a radiolabeled LFG probe. LFG is expressed in all cortical layers with the exception of layer I; in hippocampus, the highest expression was observed in CA3 and dentate gyrus (DG). Additionally, expression was also observed in piriform cortex, olfactory bulb, and the IGL and PCs of the developing cerebellum. Because LFG knockdown mice still showed some residual expression, we decided to perform ISH to evaluate the pattern of down-regulation in the brain. We observed substantial down-regulation in all of the laminar structures such as cerebellum, neocortex, hippocampus, and olfactory bulb, but it was not a uniform pattern: Greater reduction was observed in the medial to caudal most part of the cortex, whereas expression was retained in the rostral cortex; within the cerebellum, knockdown was stronger in the IGL than in the PC layer but remained significant in both layers (Fig. 2).

Cytoarchitectural Analysis. We observed that shLFG mice had a reduced cerebellar size compared with WT, and measured cerebellar volume at three different developmental stages. At postnatal day 5 (P5), shLFG cerebella were 38.8 ± 4.2% smaller than WT. By P10, the difference was slightly reduced to 28.5 ± 7.4% of WT and by P35 to 17.7 ± 3.1% of WT. These data revealed that the cerebellum of shLFG mice was significantly smaller than that of WT mice, with the biggest differences in the early developmental stages (P5). More importantly, overtime the difference was reduced from 38.8% to 17.7%, showing a substantial developmental recovery. However, even at the P35 stage in which cerebellar development is complete, shLFG mice still had a significantly smaller cerebellum. Overall the cerebellar structure and foliation were not affected (Fig. 3 *A* and *B*).

Histological analysis showed that the IGL of shLFG mice was thinner than WT, especially at P10. To quantify this observation, we measured the thickness of the IGL in two cerebellar lobules III and VI at P10 and P35. shLFG mice had an IGL that was 17.4 ± 2.6% (III) and 18.5 ± 4.3% (VI) thinner than the WT one at P10. At P35, there were no significant differences in IGL thickness between WT and shLFG mice (Fig. 3 *C* and *D*). These data indicate that loss of LFG leads to reduced cerebellar volume and density of granular cells of the IGL that is most severe at the early developmental stages. Interestingly, we observed a recovery over time, providing a remarkable example of cerebellar plasticity.

Loss of LFG Affects Purkinje Cell Differentiation and Survival. To further investigate the role of LFG in development of the PCs, sections of P5, P10, and P35 WT, shLFG, and KO brains were immunostained for the PC markers parvalbumin (PV) and calbindin (CB), two calcium-binding proteins that act as calcium buffers.

PV has been well characterized as a differentiation marker for PCs (16, 17), so we examined the level of differentiation of the PCs based on the presence or absence of PV expression. PCs from WT mice showed PV immunostaining at P5, P10, and P35. In contrast, the shLFG and KO mice showed no PV expression at P5 in the Purkinje cells even though CB immunostaining of adjacent sections demonstrated the presence of the PCs (Fig. S4). By P10, the staining was restricted to few PCs in shLFG mice, but completely recovered in the KO compared with WT mice. At P35, all strains had similar PV staining (Fig. 4*A*). These data indicate that reduction or loss of LFG causes a delay rather than a lack of PC differentiation.

We observed that PC density was reduced in adult shLFG and KO mice compared with WT. Quantification showed that shLFG mice had a 22.65% ± 6.7% reduction in Purkinje cell density (9.1 PC per field) and LFG KO mice had a 17.9% ± 5.8% reduction in PC density (9.66 PC per field) compared with WT (11.77 PC per field) (Fig. 4*B*). We also performed active caspase 8 and caspase 3 immunohistochemistry and observed a higher number of PCs positive for both markers in the shLFG and KO mice compared with WT. As expected, we observed more apoptotic PCs in the shLFG mice compared with the KO mice (Fig. 5 *A*–*F*'). Taken together, these data suggest a role of LFG in the maintenance of PCs in adult mice.

It was worth noting that some of the shLFG and KO PCs had an abnormal morphology, so we decided to investigate whether there was any correlation with Purkinje cell morphology and cell death. For this purpose, we defined a normal PC as one with a round shaped cell body (R) and an abnormal PC as one with an elongated cell body (E) or irregular plasma membrane borders (I) (see Fig. 5 *G* and *H* for examples). Then, we evaluated the morphology of the PCs and their correlation with active caspase 3 staining across all genotypes and found that 11.4% ± 1.9% (30+/263R) were active caspase 3 positive in the R group, 73.8% ± 4% (62+/84E) in the E group and 72.58% ± 3.04% (45+/62I) in the I group (Fig. 5*I*). Fig. 5 *J*–*L* are representative images of Nissl-stained PCs of WT, shLFG and KO mice, respectively, showing that although most of the PCs in the WT fall into the R category, several abnormal PC have either an elongated cell body or an irregular plasma mem-

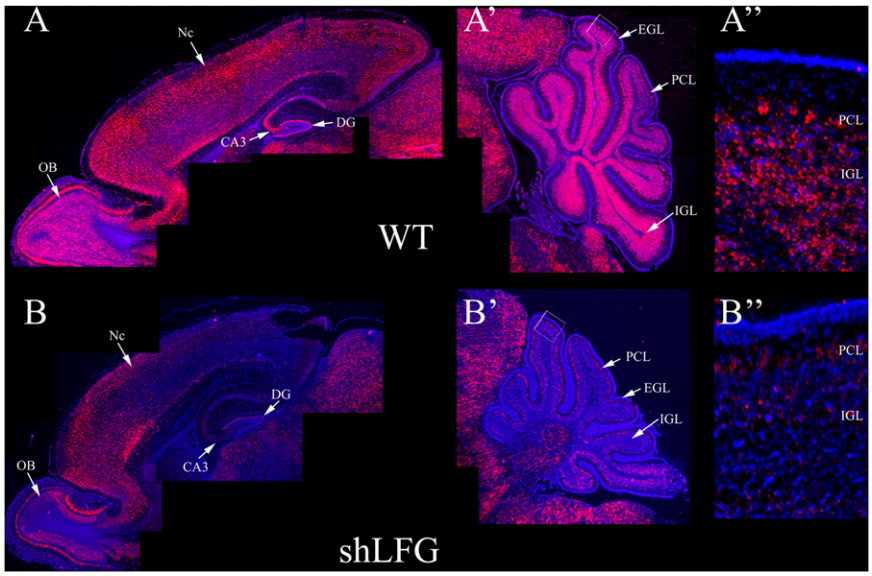

Fig. 2. Pattern of LFG expression in the CNS and its down-regulation in shLFG mice. Reconstruction of ISH of a sagittal section from P10 WT and shLFG brain, using the LFG probe (pink) and counterstained with DAPI (blue). (*A* and *B*) Telencephalon. (*A′* and *B′*) Cerebellum. (*A″* and *B″*) High-magnification insets from the cerebellar area delineated by the squares in *A′* and *B′*. Nc, neocortex; OB, olfactory bulb; DG, dentate gyrus; CA3, cornu ammonis 3; PCL, Purkinje cell layer; EGL, external granular layer; IGL, internal granular layer.

brane in the shLFG and KO mice. With these data, we show a correlation between the PC morphology and apoptosis.

Fas-Mediated Cell Death in Organotypic Cerebellar Cultures. Because LFG protects cells from Fas-mediated apoptosis, we wanted to test the effect of Fas activation in organotypic cultures from WT and LFG KO mice. For this study, we decided not to use the shLFG mice due to the inherent variability of the model. PCs have a poor survival rate in organotypic cultures from mice between P1 and P5 because this time frame represents the naturally occurring cell death period of PCs (18). The main goal of this experiment was to determine whether LFG null PCs were sensitive to Fas-mediated cell death; therefore, we decided to use mice between P8 and P12 to have less background of naturally occurring PC death and a better defined IGL and PC layers.

LFG KO and WT cerebella were sagittally sectioned at 400-μm thickness. Cerebellar slices were cultured for 5 d and then treated with the Jo2 Fas agonistic antibody to induce apoptosis. After 24 h of incubation with Jo2, the cerebellar slices were stained for the PC marker Calbindin and TUNEL to determine the level of cell death. Apoptotic cells were counted in two different fields: (*i*) the Purkinje cell field, defined by the expression of CB, and (*ii*) the internal granular layer field, adjacent to the PC field and negative for CB immunostaining (Fig. 6*A*). Untreated WT and LFG KO cells showed no major differences in cell death in the PC field; WT = 5.85 ± 1.59, KO = 10.52 ± 1.61 TUNEL-positive cells ($P = 0.153$, not significant). However, treatment with Jo2 significantly increased the number of apoptotic cells in the LFG KO PC field; WT = 4.66 ± 0.81, KO = 19.23 ± 3.21 TUNEL-positive cells ($P < 0.001$; Fig. 6 *B* and *C*) [ANOVA: genotype (WT/KO) × treatment (UT/Jo2): $F(1, 14) = 5{,}126$; $P = 0.04$]. Similar results were obtained for the IGL field with no significant differences in cell death in the untreated condition; WT = 10.67 ± 2, KO = 14.88 ± 2.79 TUNEL positive cells ($P = 0.409$, not significant) and significant effects on the survival of KO cells in the IGL field after Jo2 treatment; WT = 8.54 ± 2.65, KO = 28.73 ± 4.85 TUNEL-positive cells ($P = 0.001$; Fig. 6 *D* and *E*) [ANOVA: genotype (WT/KO) × treatment (UT/Jo2): $F(1, 14) = 5.215$, $P = 0.039$]. These data suggest that loss of LFG increases Purkinje and granular cell susceptibility to Fas-mediated cell death.

Discussion

We addressed the role of LFG in the cerebellum in vivo. We have used LFG knockdown and KO mice to investigate the function that LFG plays in cerebellar development. Our results show: (*i*) reduced cerebellar size and internal granular layer (IGL) thickness in early developmental stages; (*ii*) delayed Purkinje Cell (PC) development, with an abnormal morphology and reduced cellular

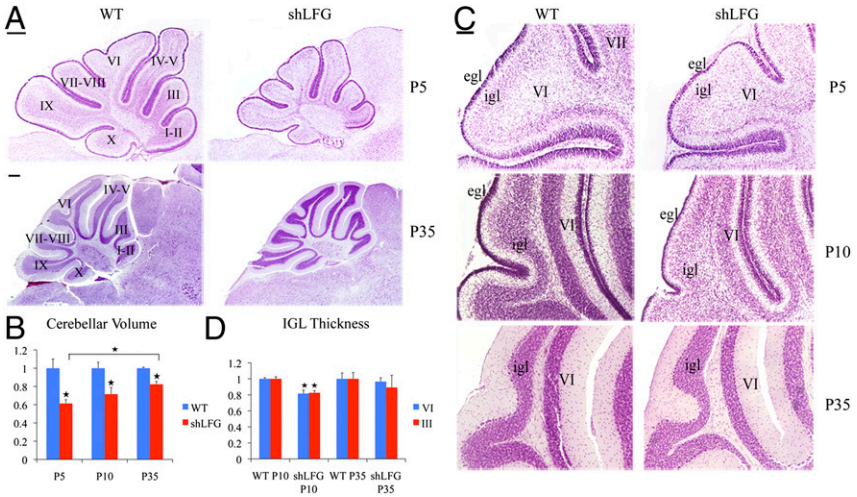

Fig. 3. Loss of LFG affects cerebellar volume and internal granular layer thickness. (*A*) Cresyl violet staining of cerebellar sagittal sections at P5 and P35 showing that shLFG cerebella are smaller than WT. (*B*) Histograms representing the quantification of cerebellar volume (μm²) of WT and shLFG mice at P5, P10, and P35. (*C*) Nissl staining of P5, P10, and P35 sagittal sections showing that shLFG mice have a reduced internal granular layer (igl, internal granular layer; egl, external granular layer). (*D*) Histograms representing the quantification IGL thickness (μm) in lobules III and VI from WT and shLFG mice at P10 and P35. *$P \leq 0.05$, **$P \leq 0.01$, ± SEM n = 3–6 mice per group. (Scale bars: 500 μm in *A* and 50 μm in *B*.)

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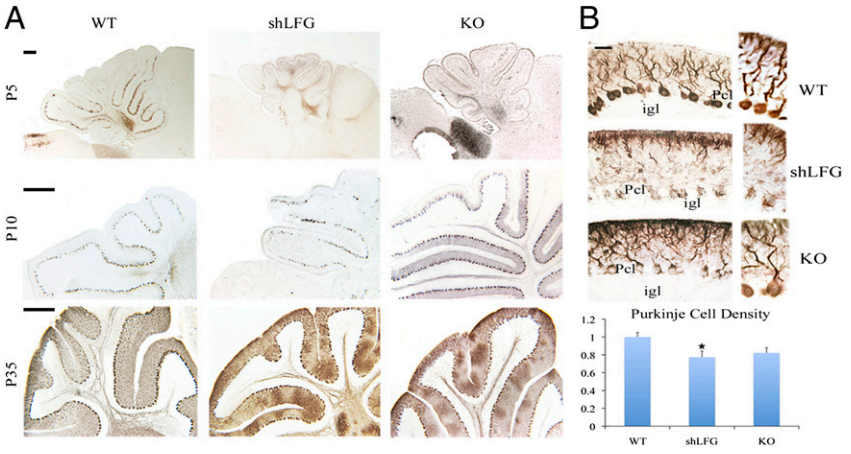

Fig. 4. Loss of LFG results in delayed PC differentiation, abnormal cell morphology and reduced cell density. (*A*) Parvalbumin immunohistochemistry of P5, P10, and P35 cerebella shows a delay in PC differentiation most severe in shLFG mice. (*B*) Parvalbumin immunostaining at P35 reveals a lower cellular density especially in shLFG mice. Histograms representing the quantification of PC density $*P \leq 0.05$, ± SEM. (Scale bars: 250 μm in *A* and 40 μm in *B*.) Pcl, Purkinje Cell layer; igl, internal granular layer.

density; (*iii*) increased caspase 8 and caspase 3 activity in PCs; and (*iv*) higher sensitivity to Fas-mediated apoptosis in LFG null organotypic cerebellar cultures. These results point to a role of LFG in mediating survival and maintenance of CGNs and PCs.

In the canonical Fas-induced apoptosis, binding of FasL to FasR is followed by recruitment of FADD. In turn, FADD interacts with the protease caspase 8 via a death effector domain (DED) common to both proteins to form the DISC. DISC formation results in autoproteolytic processing of caspase 8 and release of its active form (19). Active caspase 8, either directly or through the mitochondrial pathway, activates caspase 3, ultimately leading to cell death (20). The experiments performed here showed that LFG inhibits the Fas apoptotic pathway by interfering with caspase 8 activation but not with its recruitment to the DISC (Fig. 1). Our finding is in agreement with previous studies that reported a reduced caspase 8 activation in the presence of LFG (13, 14). How LFG interferes with the processing of the procaspase 8 remains to be determined. One possibility is that, in the presence of LFG, other proteins are recruited to the DISC along with caspase 8 and are blocking its autoproteolytic cleavage. A molecule that has been described to perform such function is the FLICE-like inhibitory protein (FLIP) (19). However, Fernandez et al. (14) showed that LFG is able to protect CGNs from Fas-mediated cell death in the absence of FLIP. Therefore, it is possible that other FLIP-like molecules containing a DED domain are recruited to the DISC and are able to block caspase 8 activation. LFG immunoprecipitated with the FasR, and, interestingly, their interaction was reduced in the presence of stimulation. Thus, it is possible that the interaction with FasR regulates LFG activity.

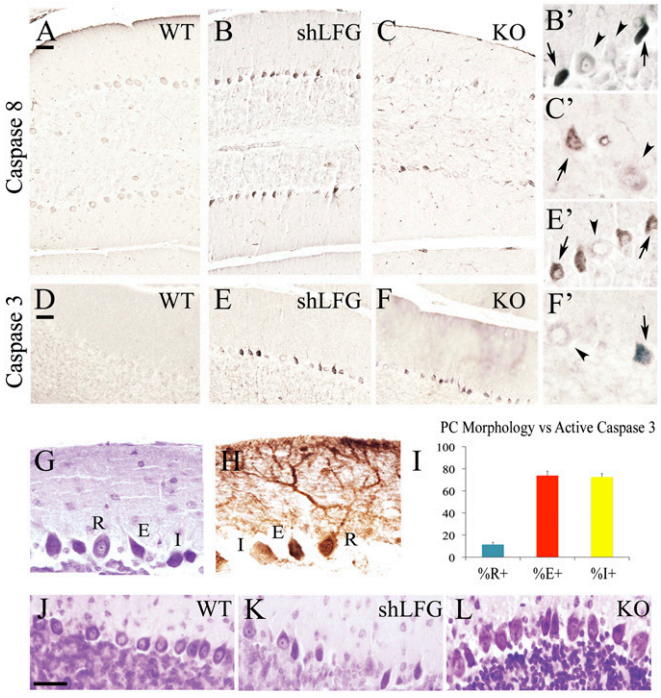

Fig. 5. Increased caspase 8 and caspase 3 activation in shLFG and KO Purkinje cells that correlates with abnormal PC morphology. (*A–C*) Representative pictures of active caspase 8 immunostaining in cerebellar sagittal sections from WT, shLFG, and KO mice at P35. (*B′* and *C′*) High-magnification insets showing positive (arrows) and negative (arrowheads) examples of active caspase 8 staining in shLFG and KO PCs. (*D–F*) Representative pictures of active caspase 3 immunostaining in WT, shLFG and KO PCs at P35. (*E* and *F′*) High-magnification insets of shLFG and KO PCs positive (arrows) or negative (arrow heads) for active caspase 3 staining. (*G* and *H*) High-power images of Nissl and Parvalbumin staining of PCs illustrating the three types of morphology: R, round ; E, elongated; I, irregular. (*I*) Histograms representing the correlation between PC morphology and active caspase 3 staining expressed in percentage. (*J–L*) Representative pictures of Nissl-stained PCs of WT (*J*), shLFG (*K*) and KO (*L*) mice. (Scale bars: 40 μm.)

PCD is involved in embryonic development, tissue remodeling, tumor surveillance, and regulation of the immune system. Dysfunction of this process can lead to serious developmental defects, oncogenesis, and autoimmune and degenerative diseases. PCD also plays an important role in the development of the central nervous system (CNS) regulating the final number of neurons and glial cells in the adult brain. Two major waves of apoptosis have been identified during CNS development in mice. The first one occurs between embryonic days E10 and E18 and is related to cell cycle regulation; it affects mainly progenitor cells and young postmitotic neuroblasts. The second apoptotic wave occurs during early postnatal stages and affects postmitotic neurons establishing neuronal connections (21). During this postnatal period, most of the cerebellar structural development takes place. PCD has been described during the development of PCs, CGNs, and deep cerebellar neurons (18, 22–24).

Our results are consistent with the antiapoptotic function of LFG. In early postnatal stages, we found that loss of LFG affected cerebellar volume, IGL thickness, and PC development. Interestingly, we observed an improvement over time showing the plasticity and recovering capacity of the brain. In adult mice, we found a lower PC density and an increase in caspase 8 and caspase 3 activity in the absence of LFG. Taken together, these data suggest a neuroprotective role for LFG in early postnatal stages affecting mostly CGNs and a maintenance role in adulthood that primarily affects PCs.

In addition to the apoptotic function of Fas, evidence for its nonapoptotic roles in neurons also exists. For instance, mice deficient for FasR or FasL showed reduced number of dendritic

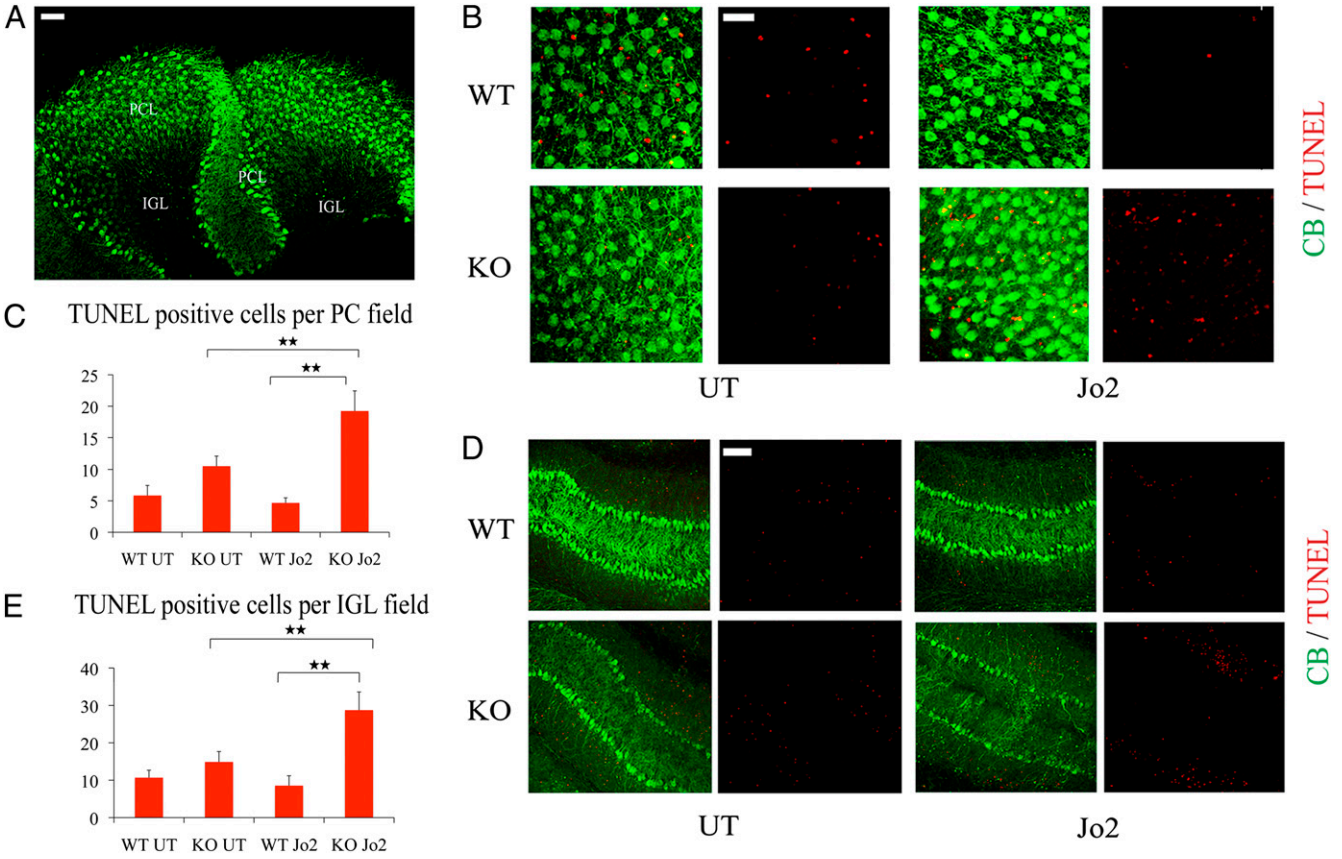

Fig. 6. LFG null cerebellar slices are more susceptible to Fas-mediated cell death. (*A*) Representative picture of a cerebellar slice immunostained with Calbindin (CB) to define the PC and IGL fields. (*B* and *D*) Representative images showing the increase in TUNEL positive cells in LFG KO PC (*B*) and IGL (*D*) fields after Jo2 treatment. Untreated (UT), CB in green and TUNEL in red. (*C*) Histograms representing the number of TUNEL positive cells per PC field. (*E*) Histograms representing the number of TUNEL-positive cells per IGL field. $*P \leq 0.05$, $**P \leq 0.01$, ± SEM $n = 4$WT (48 fields per condition) and 5KO (60 fields per condition). (Scale bars: 50 μm in *A*, 25 μm in *B*, and 50 μm in *D*.)

branches in embryonic hippocampal and cortical neurons, as well as in PCs but no difference in neuronal numbers (25, 26). Furthermore, Corsini et al. (27) recently reported a role of Fas in neurogenesis. In this respect, it would be interesting to investigate whether expression of LFG in CGNs and PCs not only protects from Fas-mediated cell death, but also allows Fas-mediated non-apoptotic functions.

Loss of LFG affected CGNs and PCs, two cell populations that depend on each other for their survival. For instance, in Reeler mice, there is a reduced proliferation of external granular cells that causes a defect in PC migration (28). Another example is the lurcher mouse, which has a gain of function of glutamate receptor ionotropic delta 2, resulting in death of PCs that lead to a secondary loss of CGNs (29). This raises the question of whether LFG affects the survival of both cell types directly or it has a primary effect in one population and a secondary effect on the other.

CGNs have been shown to be resistant to Fas-mediated apoptosis, but reduction of LFG expression by shRNA renders them sensitive to Fas-induced cell death in culture (13, 14). Taking these data into account, together with our shLFG mouse model that shows reduced IGL density, we can expect a direct effect of the down-regulation of LFG in the survival of CGNs. Now the question is if the loss of LFG has a direct effect in the survival of PCs.

The comparison between our two mouse models, the shLFG and the LFG KO mice, may shed some light into this issue. In the shLFG mice, both CGNs and PCs are affected, indicating that the PC phenotype may be a consequence of the reduced IGL size. On the other hand, the LFG KO mice showed no differences in IGL density and yet presented a developmental delay and reduced density of the PC layer, suggesting a direct effect of the loss of LFG in PCs.

The finding that the shLFG mouse exhibited a more severe phenotype than the KO mouse was unexpected, although not unique and similar to what was reported for the doublecortin or the doublecortin-like kinase KO and knockdown mice (30–32). As proposed for doublecortin, it is possible that complete deficiency of the protein induces compensatory effects that contribute to reduce the severity of the phenotype. In the case of LFG, it could be reasonable to hypothesize that such compensatory effects may include up-regulation of other antiapoptotic proteins. The possibility of off-target effects of the shRNA was also considered but was not supported by the fact that: (*i*) two independent in vitro studies have shown that CGNs loose their resistance to Fas-mediated apoptosis after down-regulation of LFG mediated by different shRNA sequences (13, 14), and (*ii*) our in vivo observations showing similar phenotypes in the shLFG and the KO mice.

The phenotype we describe here is similar to that of mice with a misexpression of the homeobox gene engrailed 2 (L7En2 mice) (16). Like shLFG mice, L7En2 mice have hypoplastic cerebellum with delayed PC differentiation. It is important to point out that engrailed is a transcription factor that regulates the expression of many other genes, although LFG might be downstream of engrailed. We know that LFG protects cells from Fas-mediated apoptosis, and this study suggests a role for both LFG and the Fas pathway in cerebellar development. We have used the organotypic cerebellar culture system to show that granular and PCs become sensitive to Fas-mediated cell death in the absence of LFG. Therefore, it is possible that the Fas pathway plays a role in the naturally occurring cell death process that takes place in the cerebellum during early development when postmitotic neurons are establishing neuronal connections. Perhaps, in this context, neu-

rons that manage to establish proper connections would express LFG as a survival signal, whereas neurons that fail to do so would die from Fas-mediated apoptosis.

Although LFG is highly expressed in cerebellum, it was also found in other brain regions. These regions include cortex, olfactory bulb, and hippocampus, which like cerebellum are laminated structures but whose development is not significantly affected by LFG. In this respect, it is important to note that cerebellar development occurs primarily postnatally and correlates with an increase in LFG expression (12). Instead, cortex or hippocampus develop during embryonic stages, when LFG's expression is lower. Therefore, although LFG appears to play a role in cerebellar development, its role in cortical and hippocampal neurons appears not to be developmental.

The significance of the central role of LFG has been supported by a recent publication describing its neuroprotective role in a mouse model of stroke (33). It will be interesting to investigate whether LFG plays a role in other brain pathologies, such as traumatic injury and neurodegenerative diseases.

Materials and Methods

Caspase 8 Activity and Cell Viability Assays. Cells were seeded in 24-well plates. Fas agonistic antibody CH11 (0.5 μg/mL) was added to half of the wells; the remaining wells were left untreated for controls. To analyze caspase 8 activity and cell viability, we harvested cells at 0, 6, 12, and 24 h after CH11 treatment. We used the Caspase-GLO 8 assay based on hydrolysis of a luminogenic substrate containing the LETD sequence specific for caspase-8 and the CellTiter-Glo assay, which quantifies ATP as a measure of metabolic activity in live cells (Promega, Madison, WI). The results are expressed as the percentage of caspase 8 activity or cell viability in the CH11 treated samples compared with their corresponding untreated controls.

Mice. LFG knockdown mice were generated by shRNA lentiviral transgenesis (15). shRNAs against LFG were designed and cloned into the U3 region of a lentiviral vector under the mouse U6 Pol III promoter. The vector also contains a PGK-driven GFP cassette to monitor transduction. Validation of the construct was carried out by transfection in 293T cells and transduction of NIE 115 neuroblastoma cells. LFG null mice were purchased from Jackson Laboratories (Bar Harbor, ME). Validation of both mouse models was performed by QPCR of cerebellar cDNA (For more details see *SI Materials and Methods* and Figs. S2 and S3).

Cerebellar Slice Cultures. For a detailed protocol see Hurtado de Mendoza et al. (34).

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