Development/Plasticity/Repair

Misplacement of Purkinje Cells during Postnatal Development in Bax Knock-Out Mice: A Novel Role for Programmed Cell Death in the Nervous System?

A-rong Jung,[1] Tae Woo Kim,[1] Im Joo Rhyu,[1] Hyun Kim,[1] Young Don Lee,[2] Sharon Vinsant,[3] Ronald W. Oppenheim,[3] and Woong Sun[1]

[1]Department of Anatomy, BK21 Program, Korea University College of Medicine, Sungbuk-Gu, Seoul 136-705, Korea, [2]Department of Anatomy, Ajou University of Medicine, Suwon 443-721, Korea, and [3]Department of Neurobiology and Anatomy and Neuroscience Program, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157

During early postnatal development, the orchestrated regulation of proliferation, migration and the survival versus elimination of neurons is essential for histogenesis of the cerebellum. For instance, Purkinje cells (PCs) promote the proliferation and migration of external granule cells (EGCs), whereas EGCs in turn play a role in the migration of PCs. Considering that a substantial number of neurons undergo programmed cell death (PCD) during cerebellar development, it seems likely that neuronal loss could have a significant role in the histogenesis of the cerebellum. To address this question, we examined postnatal development of the cerebellum in Bax-knock-out (KO) mice in which the PCD of PC has been reported to be selectively reduced or eliminated, whereas EGCs are unaffected. We confirmed the absence of PC PCD as well as the normal PCD of EGCs in Bax-KO mice. We also observed a subpopulation of PCs that were misplaced in the inner granule cell layer of Bax-KO mice on postnatal day 5 (P5) to P10 and that, by the end of the major period of cerebellar histogenesis (P14), lose expression of the PC marker calbindin. These results suggest that the removal of ectopically located neurons may be a previously unrecognized function of developmental PCD.

Key words: **Bax; programmed cell death; cerebellum; Purkinje cell; cerebral cortex; migration**

Introduction

Programmed cell death (PCD) occurs at different stages of normal nervous system development. Beginning with neurulation, the death of a subset of neural stem/progenitor cells, PCD may regulate the size of proliferating cell populations and ultimately the size of the brain (Haydar et al., 1999; Kuan et al., 2000; Depaepe et al., 2005). Postmitotic PCD occurs after the formation of provisional synaptic connections and contributes to the establishment of optimal connections between neurons and their afferent and efferent targets by eliminating excess neurons (Oppenheim, 1991; Buss et al., 2006a). The PCD of postmitotic neurons can also occur before synaptogenesis, at which time it may function to eliminate ectopically located neurons and aberrant axon projections (Clarke et al., 1998; Thanos, 1999; Oppenheim et al., 2001). The proapoptotic gene Bax is required for the normal PCD of many populations of developing postmitotic neurons (White et al., 1998; Sun et al., 2003, 2004; Buss et al., 2006b), whereas the PCD of proliferating neuronal progenitor cells is less affected by Bax deletion, suggesting that the molecular cell death machinery differs for mitotic versus postmitotic neurons.

The development of the cerebellum is a complex process that requires the rather precise orchestration of multiple biological processes, such as proliferation, migration, PCD, and differentiation of multiple cell types (Hatten and Heintz, 1995). The proliferation of external granule cells (EGCs) is promoted by factors secreted from Purkinje cells (PCs) such as sonic hedgehog (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999; Lewis et al., 2004), and the migration of PCs is regulated by factors secreted from the external granule layer (EGL) such as Reelin (Jensen et al., 2002). Therefore, the accurate migration and survival of a subset of PCs is essential for normal cerebellar development, including the generation of sufficient numbers of granule cells. The importance of PCs in these events is clearly demonstrated in Reelin null mice that exhibit reduced proliferation of EGCs in the face of aberrant PC migration (Yuasa et al., 1993; Miyata et al., 1997).

Recent reports have demonstrated that Bax-knock-out (KO) mice exhibit a substantial increase in the number of surviving PCs (i.e., reduced PCD), whereas the PCD of granule cells is unaffected (Fan et al., 2001). Observation of postnatal development of the cerebellum in Bax-KO mice in which the PCD of PCs is prevented indicates that a subset of PCs are ectopically localized in the cerebellum as the result of perturbed migration. These data suggest that, during normal development, many PCs that fail to

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Correspondence should be addressed to Dr. Woong Sun, Department of Anatomy, Korea University College of Medicine, 126-1 Anam-Dong, Sungbuk-gu, Seoul 136-705, Korea. E-mail: woongsun@korea.ac.kr.

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migrate correctly are selectively deleted by PCD, whereas in the absence of PCD (Bax deletion) these ectopic PCs are retained.

Materials and Methods

Animals. Heterozygous Bax-deficient mice were maintained on a C57BL/6 background. Sibling animals were collected and individually genotyped by PCR as described previously (Knudson et al., 1995). Bax mice were generated from matings between heterozygous (+/−) males and females. Females were examined each morning for the presence of a vaginal plug. Once a plug was detected, the female was removed from the male's cage, and noon of that day was designated as embryonic day 0.5 (E0.5). For PC birthdating analysis, pregnant mice were injected with bromodeoxyuridine (BrdU) (50 μg/g body weight) at E12 of gestation, brains were collected at postnatal day 5 (P5) or P14, and the number of BrdU-labeled PCs was evaluated as described below.

Reeler mutants with Orleans alleles (Goffinet, 1983) were maintained on C57BL/6 genetic background. Homozygotic mice were produced by the mating of heterozygote mice, and the genotypes were verified by PCR, as reported previously (D'Arcangelo et al., 1995).

Immunohistochemistry. For immunohistochemical analysis, animals were perfused with 4% paraformaldehyde in PBS, and the brains were postfixed in the same fixative for 24 h. The brains were then cryoprotected in 30% sucrose and sectioned (16 μm) parasagittally on a cryostat. For BrdU labeling, sections were incubated with 0.2N HCl for 1 h at 37°C, washed with TBS, and blocked with 3% BSA and 0.2% Triton X-100 in TBS for 30 min. The following primary antibodies were applied overnight: anti-BrdU (1:1000; Roche Diagnostics, Mannheim, Germany); anti-activated caspase-3 (1:1000; Cell Signaling Technology, Beverly, MA); anti-calbindin (CB) (1:1000; Sigma, St. Louis, MO); anti-neuronal-specific nuclear protein (NeuN) (1:1000; Chemicon, Temecula, CA); anti-GFAP (1:1000; Sigma); anti-pax6 (1:1000; Chemicon); anti-Tag-1(1:100; provided by the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); and anti-microtubule-associated protein 2 (MAP2) (1:1000; Chemicon). After several washes with PBS, appropriate secondary antibodies (Invitrogen, Carlsbad, CA) were applied for 30 min. Subsequently, the sections were washed, mounted, and observed under a fluorescence or confocal microscope (LSM510; Zeiss, Goettingen, Germany).

Electron microscopy. Mouse pups (P5) were deeply anesthetized on ice and were perfused intracardially with 40 ml of freshly made 2% glutaraldehyde, 2% paraformaldehyde in 0.13 M sodium cacodylate buffer, pH 7.4, at a flow rate of 5 ml/min. After decapitation, brains were removed and postfixed in the same fixative at 4°C overnight. Parasagittal sections were cut at 350 μm on a vibratome, and lobules 4–8 were dissected using epi-illumination. Specimens were then embedded in Araldite 502 using a Lynx processor. One micrometer sections and subsequent 700 Å thin sections were cut using an LKB (Piscataway, NJ) ultramicrotome, counterstained with toluidine blue for 1 μm sections or uranyl acetate in 100% methanol followed by lead citrate for thin sections, which were then viewed with a Zeiss EM 10 electron microscope. To verify the location of observed neurons, thin-section boundaries and landmarks were plotted onto graph paper using *x–y* stage coordinates from the electron microscope. A camera lucida was then used to project the adjacent 1 μm sections onto the graph of the thin section, and cytoarchitectural boundaries were drawn. The *x–y* stage coordinates of all electron micrographs were subsequently recorded and plotted onto the graph.

Quantification. To estimate the extent of neuronal death, activated caspase-3-immunoreactive (IR) cells were counted in every 10th serial section through an entire cerebellar hemisphere. The different cerebellar layers were identified by the density and the size of nuclei after counterstaining with Hoechst33342; the Purkinje call layer (PCL) was further confirmed by CB labeling. Birthdates of PCs were determined by quantifying the number of BrdU-positive ($BrdU^+$)/calbindin$^+$ cells on P5 and P14.

Results

Absence of postmitotic neuronal PCD in the Bax-KO cerebellum

At the peak period of PCD in the cerebellum on P5, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL)-positive or activated caspase-3-IR cells were widespread in the EGL, the PCL, and in the marginal zone/white matter region (MZ/WM) of wild-type (WT) mice. In contrast, Bax-KO mice exhibit greatly reduced PCD in the PCL and MZ/WM (Fig. 1) (PCL: WT, 106 ± 14; KO, 8 ± 3; WM/MZ: WT, 3768 ± 292, KO, 380 ± 94; $n = 5$; $p < 0.05$ in Student's t test comparisons), whereas activated caspase-3$^+$ cells were frequently seen in the EGL, similar to WT littermates (WT, 1358 ± 218 vs KO, 892 ± 247; $n = 5$; $p > 0.05$ in Student's t test comparisons). This is consistent with previous reports demonstrating that progenitor cells in the EGL undergo PCD by a Bax-independent pathway (D'Sa-Eipper et al., 2001). Double-immunofluorescent labeling of activated caspase-3 and postmitotic neuronal (NeuN) or PC (CB) markers failed to detect double-labeled cells in Bax-KO mice, consistent with the absence of postmitotic neuronal apoptosis, although we cannot entirely exclude the possibility that there is caspase-independent, non-apoptotic PC death.

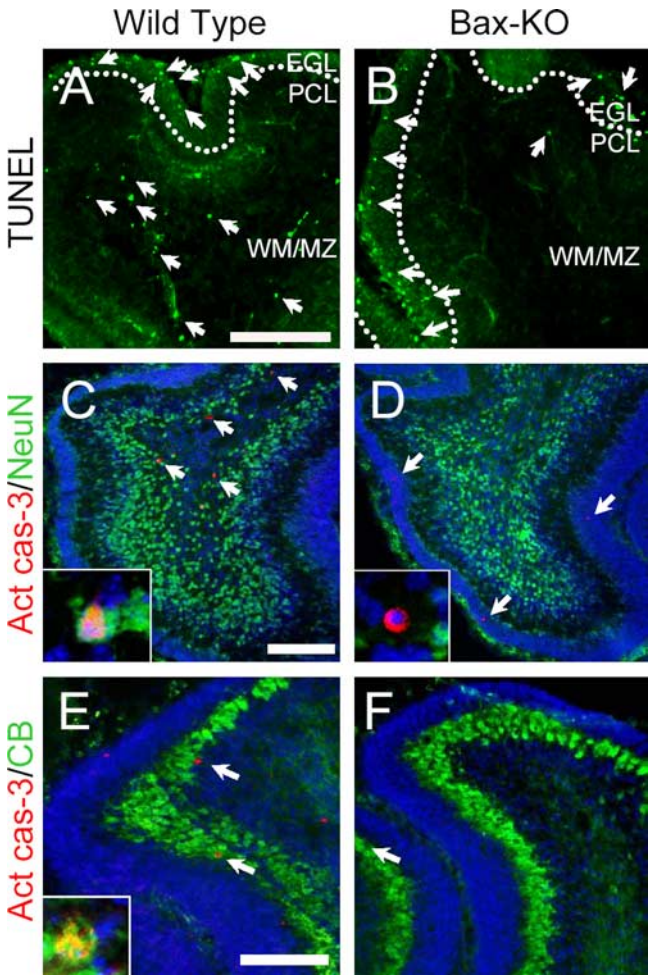

Figure 1. Region-dependent reduction of neuronal PCD in the developing Bax-KO cerebellum on P5. Typical images of TUNEL (arrows indicate TUNEL$^+$ cells; ***A***, ***B***), activated caspase-3 (arrows, red)/NeuN (green) double labeling (***C***, ***D***), and activated caspase-3 (arrows, red)/CB (green) labeling (***E***, ***F***) in sections of WT (***A***, ***C***, ***E***) and Bax-KO (***B***, ***D***, ***F***) cerebellum. Arrows in ***C*** and ***E*** indicate activated caspase-3 and NeuN (***C***) or calbindin (***E***) double-labeled WT cells in the MZ/WM, and arrows in ***D*** and ***F*** indicate activated caspase-3-labeled Bax-KO cells in the EGL. Dotted lines in ***A*** and ***B*** indicate the borders of EGL and PCL. Scale bars, 100 μm.

Misplacement of a subset of Purkinje cells in developing Bax-KO cerebellum

To examine whether the cerebellum of Bax-KO mice develops normally in the absence of PCD, we compared the general cyto-

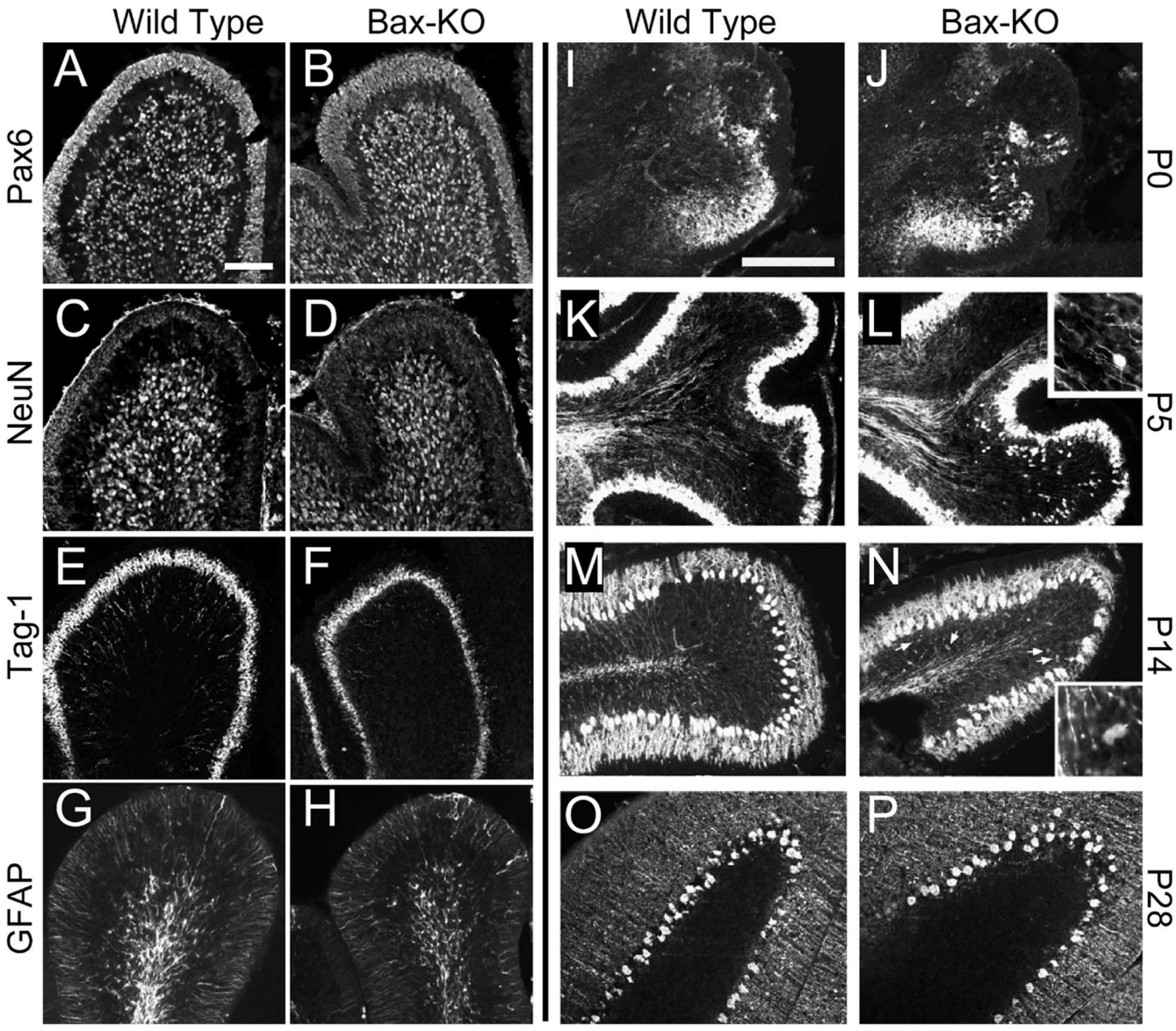

Figure 2. ***A–H***, Distribution of markers for immature neuroblasts (Pax6; ***A***, ***B***), postmitotic neurons (NeuN; ***C***, ***D***), early differentiating dendrites of Purkinje cells (Tag-1; ***E***, ***F***), and glial cells (GFAP; ***G***, ***H***) in P5 WT (***A***, ***C***, ***E***, ***G***) and Bax-KO (***B***, ***D***, ***F***, ***H***) cerebellum. ***I–P***, CB labeling on P0 (***I***, ***J***), P5 (***K***, ***L***), P14 (***M***, ***N***), and P28 (***O***, ***P***) cerebellum of WT (***I***, ***K***, ***M***, ***O***) and Bax-KO (***J***, ***L***, ***N***, ***P***) mice. Note that a subset of Bax-KO CB$^+$ cells that are atrophied is located in the IGL or deep layers at P5–P14 (***L***, ***N***, and insets). Arrows in ***N*** indicate the misplaced cells with weak CB labeling. Scale bars, 100 μm.

architecture of WT and Bax-KO cerebellum. In Nissl-stained sections, however, the general morphology of the cerebellum structure appeared normal (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Next we asked whether a distribution of markers for proliferating/differentiation granule cells (Pax6), postmitotic neurons (NeuN), PCs (CB), early developing dendrites of PCs (Tag-1), or glial cells (GFAP) at P5 were perturbed. Although the distribution of cells that were IR for Pax6, NeuN, Tag-1, and GFAP was similar in WT and Bax-KO cerebellum, there was a small subpopulation of CB$^+$ cells located ectopically in the deeper regions of the internal granule layer (IGL) in the Bax-KO (Fig. 2). In an attempt to determine the source of these ectopic cells, we examined the cerebellum in Bax-KO mice at earlier stages. At P0, both WT and Bax-KO mice exhibited a normal formation of the Purkinje cell plate and a similar distribution of CB$^+$ PCs. By P5, however, a larger subpopulation (8.4 $\pm$ 1.2%; $n = 4$) of CB$^+$ cells were ectopically localized in the IGL of Bax-KO compared with WT mice (2.2 $\pm$ 0.4%; $n = 4$; $p < 0.05$ in Student's t test comparisons). These ectopic CB$^+$ cells appeared to be smaller than CB$^+$ PCs localized in the PCL of both WT and Bax-KO mice, and, although they had a bipolar morphology with an axonal projection directed toward the white matter (Fig. 2, inset), these cells did not exhibit the typical PC dendritic arborization associated with Tag-1 immunoreactivity, suggesting that they may represent an immature stage of PC development. By P14, these misplaced Bax-KO CB$^+$ cells were still present and were intermingled with granule cells in the IGL, although the intensity of CB labeling was substantially reduced (Fig. 2, arrows and inset). By P28, the ectopic cells could no longer be identified by CB labeling, and accordingly the distribution of CB labeling was now virtually indistinguishable between WT and Bax-KO mice.

To further identify the fate of the ectopic CB$^+$ cells in the Bax-KO cerebellum, we labeled a subset of PCs by pulse labeling with BrdU on E12 of gestation. Because PCs (but not other cerebellar cells) are generated between E11 and E13 (Hatten and

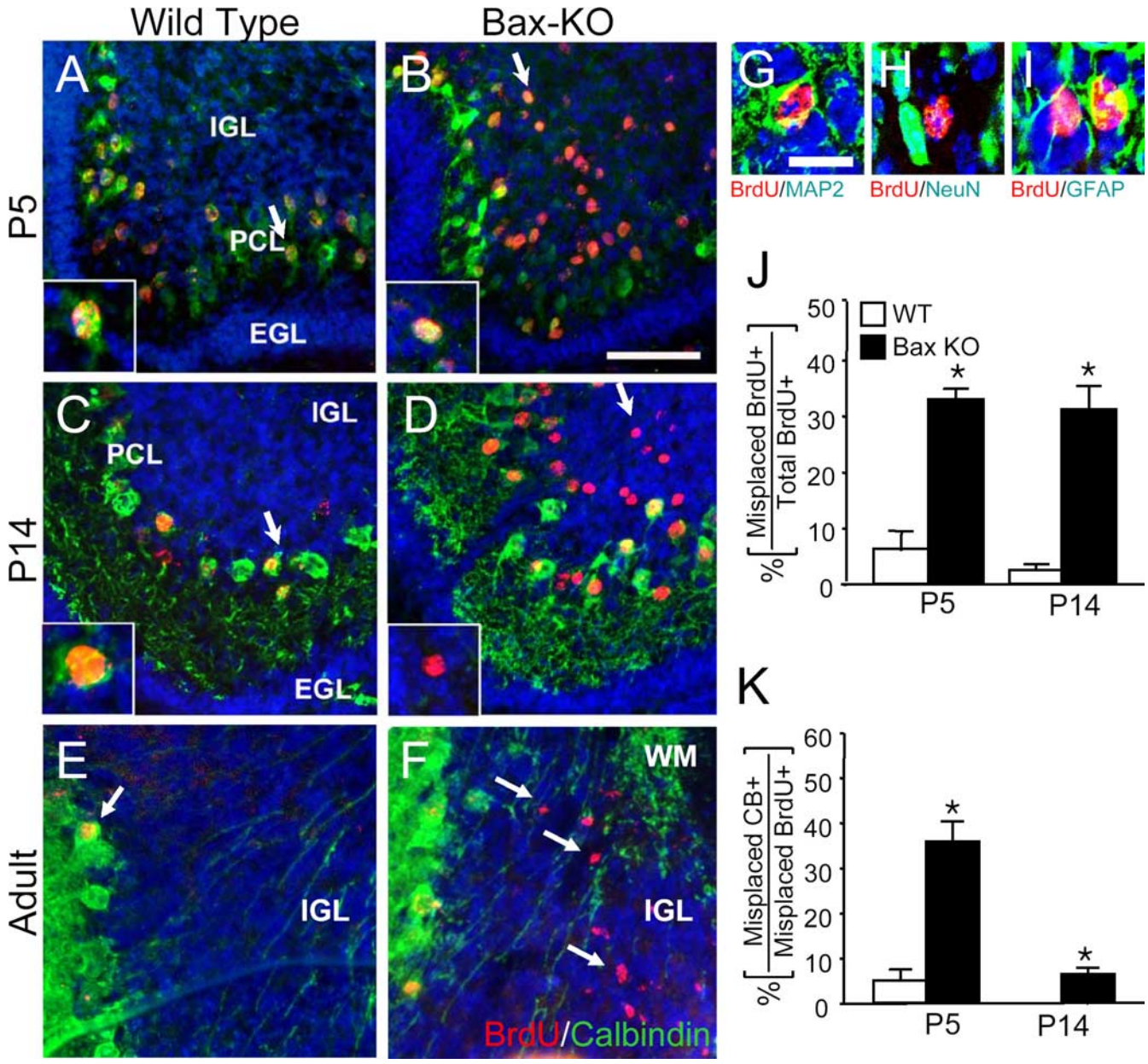

Figure 3. ***A–F***, BrdU birthdate labeling. Pregnant mice received BrdU (50 μg/g body weight) at E12 of gestation, and the distribution of BrdU-labeled cells (red) in the P5 (***A***, ***B***), P14 (***C***, ***D***), or 3-month-old adult (***E***, ***F***) cerebellum of WT (***A***, ***C***, ***E***) and Bax-KO (***B***, ***D***, ***F***) were analyzed after coimmunolabeling of CB (green) and counterstaining of the nucleus with Hoechst33342 (blue). Arrows indicate single- or double-labeled cells. High-magnification images of indicated cells are shown in insets in ***A–D***. Scale bar, 100 μm. ***G–I***, Double-immunofluorescence labeling of BrdU-labeled (red) misplaced cells with MAP2 (green, G), NeuN (***H***), and GFAP (***I***) in P14 Bax-KO cerebellum. Nuclei were counterstained with Hoechst33342 (blue). Scale bar, 10 μm. ***J***, ***K***, Quantification of the misplaced BrdU$^+$ cells per total BrdU$^+$ cells (***J***) and misplaced CB$^+$/BrdU$^+$ cells per total misplaced BrdU$^+$ cells (***K***) in P5 and P14 cerebellum. Data are expressed as mean ± SE; $n = 4$. *$p <$ 0.05 in Student's *t* test comparisons.

Heintz, 1995), the single injection of BrdU to pregnant mice on E12 of gestation selectively labeled a subpopulation of PCs. We examined the distribution and differentiation of BrdU$^+$ cells in the postnatal cerebellum of WT and Bax-KO mice (Fig. 3). Approximately 95% of all BrdU$^+$ cells in the WT cerebellum were correctly localized in the PCL, and most of these were CB$^+$. In contrast, >30% of the total number of BrdU$^+$ cells in the Bax-KO was located outside of the PCL (Fig. 3*J*). Between P5 and P14, ectopic BrdU$^+$ cells persisted, but, by P14, the number of these that were also CB$^+$ was greatly reduced (Fig. 3*K*). In the adult Bax-KO cerebellum, BrdU$^+$ cells were only infrequently seen in the deep region of the IGL or WM, and none of them were CB$^+$, suggesting that a large proportion of misplaced PCs maintain BrdU labeling but lose CB immunoreactivity in adulthood (Fig. 3*E*,*F*). Consistent with these data, we also failed to detect ectopic caspase-3$^+$ or TUNEL$^+$ cells in the Bax-KO cerebellum at any of the time points we examined (data not shown).

To further examine whether the misplaced CB-negative, BrdU$^+$ PCs maintain a neuronal phenotype on P14, we performed double-immunofluorescence labeling for BrdU with MAP2, NeuN, or GFAP (Fig. 3*G–I*). BrdU$^+$ cells exhibited MAP2 labeling, but none of them expressed GFAP, indicating that these cells maintain a neuronal phenotype. Interestingly, the misplaced BrdU$^+$ cells did not exhibit NeuN immunoreactivity, consistent with the fact that PCs do not normally express NeuN (Mullen et al., 1992).

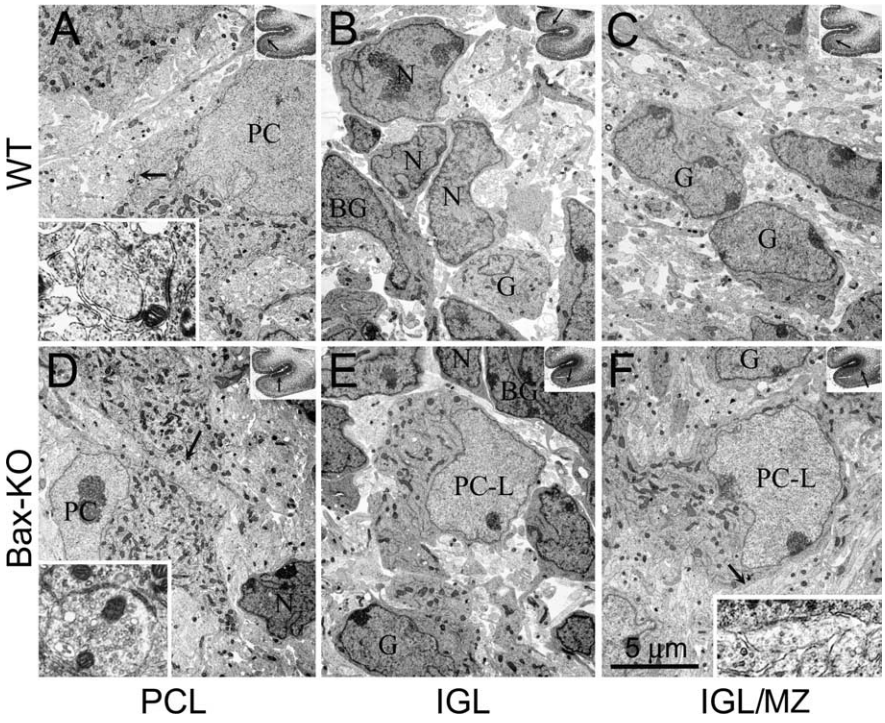

Figure 4. Morphology of the PCL (***A***, ***D***), IGL (***B***, ***E***), and deeper IGL/MZ (***C***, ***F***) of WT (***A–C***) and Bax-KO (***D–F***) cerebellum. PCs in the WT and Bax-KO PCL were indistinguishable, and they formed synapses with climbing fibers (insets in ***A*** and ***D***). Arrows in ***A*** and ***D*** point to the location of synapses that are enlarged in the insets. Migrating neuroblasts (N) descending along Bergmann glia (BG) toward the IGL and differentiating granule cells (G) were commonly found in the IGL and IGL/MZ of WT and Bax-KO mice. In the Bax KO mice, PC-Ls were occasionally found in the IGL and IGL/MZ, and they exhibited immature synapses (inset in ***F***); an arrow indicates the location of the synapse in ***F*** that is enlarged in the inset. Three animals in each group were examined, and typical images are shown. Each figure contains an inset in the top right corner showing the adjacent 1 μm section, with an arrow indicating the location of the electron micrograph. Synaptic enlargements, 20,000×.

Ultrastructural observation of the misplaced PCs in Bax-KO cerebellum

We next evaluated the ultrastructural morphology of the misplaced PCs in P5 Bax-KO cerebellum (Fig. 4). The EGL of WT and Bax-KO mice was indistinguishable, and both exhibited packed neuroblasts usually surrounded by characteristic extracellular spaces, likely representing a mixture of granule cells and stellate/basket cells (data not shown). The PCL of both WT and Bax-KO mice similarly contained large PCs characterized by smoothly dispersed chromatin in a large often crenulated nucleus and with plentiful cytoplasm containing many organelles. Synapses that likely are derived from climbing fibers were also sparsely distributed in the surrounding neuropil. In the IGL, migrating granule cells descending along Bergmann glia were commonly observed in both WT and Bax-KO mice. In addition to these aggregates of granule cells, PC-like cells (PC-Ls) were occasionally observed in the Bax-KO IGL. Whereas granule cells in the IGL do not form synapses, these PC-Ls exhibited immature synapses, providing additional support that they are misplaced PCs. Similarly, in the IGL/MZ, most cells in WT mice were differentiating granule cells with sparse cytoplasm and chromatin aggregates in the nucleus, whereas PC-Ls often with synapses were sometimes observed in the Bax-KO.

Relationship between the impaired migration and PCD of PCs in wild-type and *Reeler* cerebellum

The above observations suggest that cells that undergo aberrant neuronal migration during normal development may be eliminated by PCD. To address this possibility, we examined whether WT PCs undergo PCD when located in ectopic positions. Approximately 2% of WT CB^+ PCs are in an ectopic location on P2–P5. On P2, caspase-3-IR cells were infrequently found at the border of the PCL that exhibited reduced CB immunoreactivity (Fig. 5). Quantification of the proportion (percentage) of activated caspase-3-IR cells that are CB-IR in the PCL and in ectopic positions demonstrated the more than fivefold higher PCD in the ectopic position compared with the PCL (PCL, $1.5 \pm 0.6\%$; ectopic position, $10.4 \pm 2.6\%$; $n = 3$; $p < 0.05$ in Student's t test comparisons). On P4, when the PCL became more discrete, we also found caspase-3-IR cells below the PCL, but most of these cells did not exhibit CB immunoreactivity. Because it is possible that these cells lose CB immunoreactivity before the expression of caspase-3, we examined whether they are $BrdU^+$ after E12.5 birthdate labeling. Although it was not possible to quantify these observations, we were able to detect a few $BrdU^+$/caspase-3^+ (but CB-negative) cells close to but apparently outside of the PCL on P4, suggesting that at least a subset of ectopic WT PCs undergo PCD with progressive loss of CB expression.

To further explore the relationship of PCD with altered neuronal migration, we also examined *Reeler* mutant mice (Fig. 5*J*,*K*). Because *Reeler* mice have a mutation in the *reelin* gene, which is critical for the migration of PCs in the cerebellum, PCs fail to establish a PCL and instead are localized in deep regions of the cerebellum. In P5 *Reeler* mutants, we found a marked increase of PCD of PCs compared with the WT mice (WT, $0.5 \pm 0.1\%$; *Reeler*, $1.7 \pm 0.3\%$; $n = 4$; $p < 0.05$ in Student's t test comparisons). This observation is consistent with previous reports that the number of PCs in adult *Reeler* mice is ~50% less than WT littermates (Mikoshiba et al., 1983; Heckroth et al., 1989).

Discussion

Bcl-2 family members play essential roles in the control of PCD during nervous system development (Zanjani et al., 1996; Merry and Korsmeyer, 1997; Vekrellis et al., 1997; Vogel, 2002). The proapoptotic gene Bax appears to be especially critical for postmitotic neuronal PCD. Bax-KO mice exhibit a virtually complete absence of PCD of most populations of neurons in the CNS and peripheral nervous system (Deckwerth et al., 1996; White et al., 1998; Lentz et al., 1999; Sun et al., 2003, 2004). In contrast to postmitotic neurons, however, the PCD of mitotically active neuronal precursors is less dependent on Bax. The PCD of neuronal progenitor cells located in the subventricular zone is only marginally affected by Bax deletion (Lindsten et al., 2000, 2003; Shi et al., 2005). Proliferating neuronal progenitor cells undergo PCD via a p53-dependent, Bax-independent pathway (D'Sa-Eipper et al., 2001). In the present study, we also found no apparent reduction in activated caspase-3^+ cells in the Bax-KO EGL, which contains proliferating neuroblasts, whereas the PCD of PCs and other postmitotic neuronal populations was absent or greatly reduced by Bax deletion. This is consistent with previous obser-

vations that Bax immunoreactivity is increased during postnatal development of the cerebellum (Vekrellis et al., 1997) and that there is a selective reduction of TUNEL$^+$ cells in the cerebellar MZ/WM of Bax-KO mice (White et al., 1998). Although we did not directly determine whether the number of surviving PCs was increased in the Bax-KO mice, it has been reported previously that adult Bax-KO mice contain ~30% more calbindin-labeled PCs in the PCL (Fan et al., 2001). In view of the results presented here showing that a subset of PCs are located outside of the PCL and fail to maintain calbindin immunoreactivity at later postnatal ages, it is likely that the number of surviving PCs in the Bax-KO cerebellum may, in fact, be considerably larger than previously recognized. However, it is not clear whether Bax deletion rescues all PCs from PCD. In *Lurcher* mutant mice, PCs die via multiple mechanisms, including an autophagic pathway, and Bax deletion fails to prevent *Lurcher* mutation-induced PCD of PCs (Doughty et al., 2000; Selimi et al., 2000, 2003). It has also been reported that microglial activation is involved in promoting the PCD of PCs *in vitro* (Marin-Teva et al., 2004). Together, these data suggest that, depending on conditions, the PCD of PCs may be regulated by different molecular pathways.

After the rescue of PCs from PCD by Bax deletion, we observed that a subset of PCs are misplaced in deep regions of the IGL, most likely attributable to the impairment of PC migration. These neurons were ultrastructurally normal and formed synaptic contacts with other neurons. Additionally, we also observed that a subset of cerebral cortical neurons exhibited delayed migration in Bax-KO mice (W. Sun, unpublished observation). In a previous study, we found that Bax deletion rescues adult-generated dentate gyrus granule cells from PCD and that, in this situation, some granule cells also fail to migrate correctly and end up ectopically positioned within the hilus (Sun et al., 2004). Together, these various lines of evidence suggest that some neurons that fail to migrate properly may be selectively removed by PCD. Several mouse mutants with PC migratory defects exhibit increased PCD of PCs (Heckroth et al., 1989; Maricich et al., 1997; Croci et al., 2006). For example, disruption of the helix–loop–helix transcription factor EBF2COE2 results in increased PCD of PCs attributable to the perturbation of PC migration (Croci et al., 2006). *Reeler* mice exhibit a 50% increased loss of PCs (Heckroth et al., 1989), and we also observed a substantial increase in the number of caspase-3$^+$ PCs on P5 in the *Reeler* cerebellum. Therefore, Bax-dependent PCD may function to eliminate migration-defective neurons in these mutant mice. Supporting this idea, we found that at least a subset of PCs located on the border of or below the PCL undergo PCD in normal WT brain. Similarly, it is known that a subset of cortical neurons undergo PCD during their migration (Blaschke et al., 1996). We also observed substantial PCD of migrating neuroblasts in the rostral migratory stream of adult WT mice (Kim et al., 2007), which is absent or significantly reduced in Bax-KO mice (Shi et al., 2005). Together, these

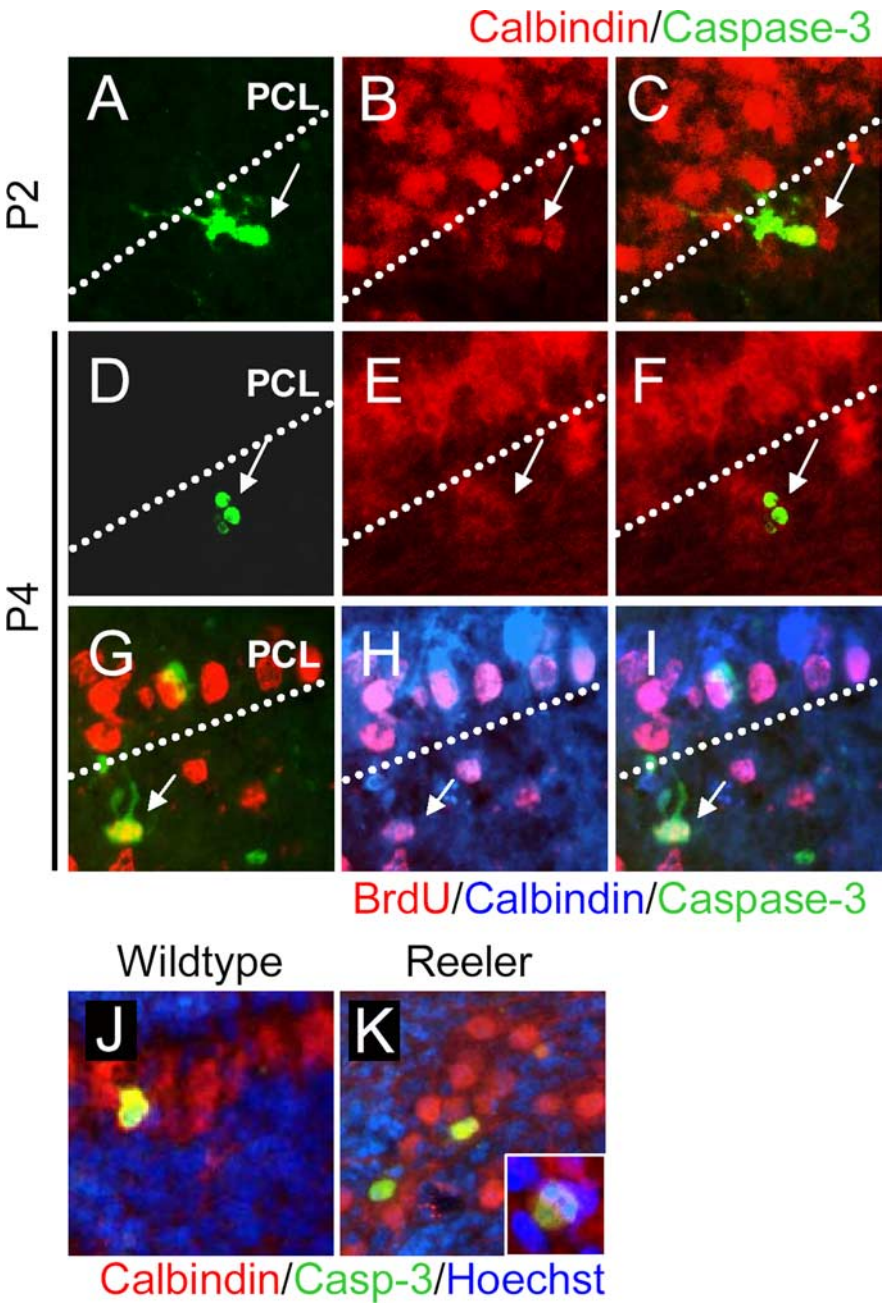

Figure 5. PCD of misplaced PCs in developing WT and *Reeler* cerebellum. ***A–F***, Double-immunofluorescence labeling for calbindin (red) and activated caspase-3 (green) on P2 (***A–C***) and P4 (***D–F***) in WT cerebellum. Activated caspase-3-IR cells (arrows) were occasionally found in the granule cell layer (GCL), and a subset of them expressed CB immunoreactivity on P2, but most caspase-3-IR cells on P4 were CB negative. ***G–I***, Triple-immunofluorescence labeling of BrdU (red), calbindin (blue), and activated caspase-3 (green) in P4 WT cerebellum. A subset of E12.5 BrdU-labeled cells in the GCL exhibit caspase-3 immunoreactivity, suggesting that these misplaced PCs progressively loss their CB immunoreactivity. Dotted lines indicate the border between the PCL and GCL. ***J***, ***K***, Enhanced PCD of PCs in *Reeler* mutant mice. CB$^+$/caspase-3$^+$ cells were increased in P5 *Reeler* mutant (***K***) compared with WT littermates (***J***). Note that caspase-3-IR cells exhibit nuclear condensation revealed by Hoechst33342 nuclear counterstaining (blue), which is another hallmark of neuronal apoptosis (inset, ***K***).

data suggest that such migration errors may be a common occurrence during normal nervous system development and that, in some cases, these errors are deleted by Bax-dependent PCD.

Although impairment of neuronal migration may be corrected by PCD, not all migration-defective neurons undergo PCD (Jacobson, 1991). For instance, adult *Reeler* mice maintain migration-impaired PCs in ectopic locations. Similarly, migration defects of cortical neurons induced by ultrasonic exposure in embryonic rats does not result in marked PCD, and most of the misplaced neurons survive until adulthood in ectopic positions (Ang et al., 2006). Therefore, although a subset of misplaced neurons are eliminated by PCD, some misplaced neurons appear to adapt to their new environment, develop an appropriate neuronal phenotype (McConnell, 1995), and establish proper target connections (Caviness and Yorke, 1976; Simmons et al., 1982). Considering that target-derived neurotrophic signals are essential for the survival of neurons after forming provisional synaptic connections, it is possible, as first suggested by Jacobson (1991), that ectopically located neurons may be at a competitive disadvantage in this regard, resulting in an increased, but not inevitable, likelihood of PCD dependent on whether they establish appropriate efferent and afferent synaptic connections. A more comprehensive analysis of the cause and the fate of mislocated neurons in the developing nervous system may help shed light on the possible role of PCD in their elimination.

An alternative explanation of our results that we cannot entirely exclude is that many of the excess rescued PCs in the Bax-KO mice may be unique in their loss of migratory ability (e.g., a pleotropic effect of loss of the Bax gene) and therefore that the situation in the Bax-KO may not reflect the occurrence of migratory errors in WT mice. For example, in previous studies, we found that a subset of excess motoneurons rescued from PCD cannot maintain their original phenotype and either become atrophied or differentiate abnormally (Sun et al., 2003; Buss et al., 2006b). In the present study, we found that misplaced PCs also exhibit an atrophied morphology and limited differentiation with eventual loss of a biochemical marker (CB) for PCs. Therefore, it is possible that the deficits in neuronal migration in Bax-KO mice and their elimination by PCD are not representative of normal migratory errors in WT mice that are removed by PCD but rather reflects yet another manifestation of altered differentiation resulting from the presence of excess neurons (i.e., PCs). Although our current experimental model does not allow us to distinguish between these two possibilities, based on the data presented here, we favor the role of PCD as a normal mechanism for the removal of a subset of misplaced neurons.

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