Supporting Information

Oishi et al. 10.1073/pnas.0808400106

SI Methods

Mouse Strains. We used 2 lines of mice expressing Cre recombinase under the control of the nestin promoter/enhancer to obtain *Pdk1* conditional knockout mice: the nestin second-intronic enhancer (1) and the 2.5-kb fragment of the nestin promoter (2). Both *Pdk1* conditional knockout mice exhibited essentially same phenotypes in the neocortex and basal ganglia. However, size reduction of the olfactory bulb was only observed by using the latter line, presumably because Cre recombinase is expressed earlier and more efficiently. Pregnant ICR mice were purchased from CLEA Japan.

Immunohistochemistry. Embryos were fixed for 3 to 8 h in PBS (PBS) containing 4% paraformaldehyde (PFA), incubated overnight at 4 °C with 30% (wt/vol) sucrose in PBS, embedded in OCT compound (Sakura Finetek), and cut with a cryostat to yield 10-μm-thick coronal sections. For detection of GABA, mice were first perfused with PBS containing 4% PFA and 0.1% glutaraldehyde (3). Immunohistochemistry was performed with antibodies to phosphorylated Akt substrates, cleaved caspase-3 (Cell Signaling), GABA (Sigma), calbindin, DARPP32, ChAT (Chemicon), Dlx1 (kindly provided by M. Nakafuku), Gsh2 (kindly provided by K. Campbell), Tbr1 (kindly provided by R. F. Hevner), NPY (ImmunoStar) or Olig2 (R&D Systems). Immune complexes were detected with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary (Molecular Probes).

Primary Culture of Neuroepithelial Cells and NPCs. Primary neuroepithelial cell cultures were prepared as described (4, 5). To obtain an NPC-enriched cell population, we plated the dissociated neuroepithelial cells on noncoated dishes in culture medium containing FGF-2 (20 ng/mL) and EGF (20 ng/mL) and cultured the cells for either 3 or 6 days with 1 passage. The resulting cell aggregates (neurospheres) were then dissociated with 0.05% trypsin and plated on poly-D-lysine-coated dishes. NPCs were further selected by retroviral infection, which takes place preferentially in dividing cells. To examine the effect of IGF-1 and Akt on the levels of Mash1 protein, insulin was removed from the NPC culture medium (by the use of N2 supplement without insulin) to lower the basal level of Mash1 protein.

Generation of Recombinant Retroviruses. Human cDNAs for a constitutively active form of Akt, which lacks the pleckstrin homology domain (residues 4 to 129) and contains an added myristoylation site, Akt3A (K179A/T308A/S473A), or AktKA (K179A) were inserted into the *Bam*HI restriction site of pMX-IRES-EGFP (kindly provided by T. Kitamura). Recombinant retroviruses were obtained as described (5).

Immunocytochemistry. Cells were fixed for 15 min at room temperature with 4% PFA in PBS, permeabilized for 30 min with 0.5% Triton X-100, and incubated overnight at 4 °C with primary antibodies. For detection of GABA, cells were fixed with 4% PFA and 0.1% glutaraldehyde. Primary antibodies included those to βIII-tubulin (TuJ1, Covance), GFP [rabbit (MBL) or chicken (Chemicon or Abcam)], Tbr1 (kindly provided by R. F. Hevner), MAP2, GABA (Sigma) and NeuN (Chemicon). Immune complexes were detected with Alexa Fluor-conjugated secondary antibodies.

Immunoblot Analysis, Immunoprecipitation, and In Vitro Kinase Assay. Brain tissue or cells were lysed as described (5). The lysates were then subjected to immunoblot analysis or immunoprecipitation. Blots were probed with antibodies to PDK1 (E3), Akt phosphorylated on Thr-308 (Santa Cruz Biotechnology), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GAD67 (Chemicon), phosphorylated glycogen synthase kinase (GSK) 3, Akt, Akt phosphorylated on Ser-473, phosphorylated Akt substrates (Cell Signaling), Mash1 (BD PharMingen), βIII-tubulin (Covance), or VGLUT1 (Synaptic Systems). Immune complexes were detected with an ECL kit (Amersham Pharmacia). Akt was immunoprecipitated and its enzymatic activity measured with an Akt kinase assay kit (Cell Signaling).

Luciferase Reporter Assay. The plasmids pKE-βA-Luc (which contains 7 repeats of the E-box motif upstream of the β-actin gene promoter and the firefly luciferase gene) and pEFBos-rat Mash1 were kindly provided by R. Kageyama and M. Nakafuku, respectively. Cells were transfected for 24 h with a firefly luciferase reporter plasmid together with various expression vectors and a *Renilla* luciferase plasmid (phRG-TK, Promega) with the use of Lipofectamine 2000 (Invitrogen). The luciferase activities of cell lysates were then measured with a Dual-Luciferase reporter assay system (Promega), and firefly luciferase activity was normalized on the basis of *Renilla* luciferase activity.

Quantitative RT-PCR Analysis. Total RNA extracted from cells with the use of a Nucleospin kit (Macherey-Nagel) was subjected to RT with an oligo(dT)$_{12–18}$ primer (Invitrogen). The resulting cDNA was subjected to real-time PCR in a Roche LightCycler instrument with SYBR-green Realtime PCR Master Mix (TOYOBO). The abundance of target mRNAs was normalized relative to that of *Gapdh* mRNA. The sense and antisense primers, respectively, were as follows: *Gapdh*, 5′-CATTGACCTCAACTACATGG-3′ and 5′-TTGCCCACAGCCTTGGCAGC-3′; *Vglut1*, 5′-TGGCTAAGGAGCTAAGTCTATG-3′ and 5′-CAGTTGAACTGGGCTTTCT-3′; *Gad65*, 5′-ACCAAGACATTGACTTCCTTATT-3′ and 5′-CATTTACACAGTTTGTGACAACTTAG-3′; and *Mash1*, 5′-CTACGACCCTCTTAGCCC-3′ and 5′-TCCTGCTTCCAAAGTCCAT-3′.

In Situ Hybridization. In situ hybridization on frozen sections was performed as described previously (6). Probes for *Vglut1*, *Lhx6*, and *Sp9* were prepared from the FANTOM clone set (7).

In Utero Electroporation. Introduction of plasmid DNA into neuroepithelial cells of embryos in utero was performed as described (8). Plasmid DNA [pCAG-IRES-EGFP (pCAGIG, kindly provided by T. Matsuda and C. L. Cepko) or pCAGIG-active Akt, at 5 mg/mL] was injected into the lateral ventricle of each littermate at E12.5. Focal electroporation was performed as described (9).

Degradation Analysis of Mash1 Protein. Cos-1 cells grown in DMEM containing penicillin-streptomycin and 10% FBS were transfected with various plasmids for 24 h. The cells were then treated with 80 μg/mL cycloheximide (Sigma) for the indicated times, lysed, and subjected to immunoblot analysis.

BrdU Incorporation Assay. Female mice on day 16.5 of gestation were injected i.p. with BrdU (400 mg/kg body mass) and killed

30 min later. The embryos were isolated, fixed with 4% paraformaldehyde, and embedded in OCT compound. After antigen retrieval by autoclave treatment for 5 min at 105 °C in 10 mM sodium citrate buffer (pH 6.0), coronal brain sections were subjected to immunohistochemistry with antibodies to BrdU (Becton Dickinson). The number of BrdU-positive cells in the VZ and SVZ of the lateral ganglionic eminence was counted in 4 serial sections of each embryo.

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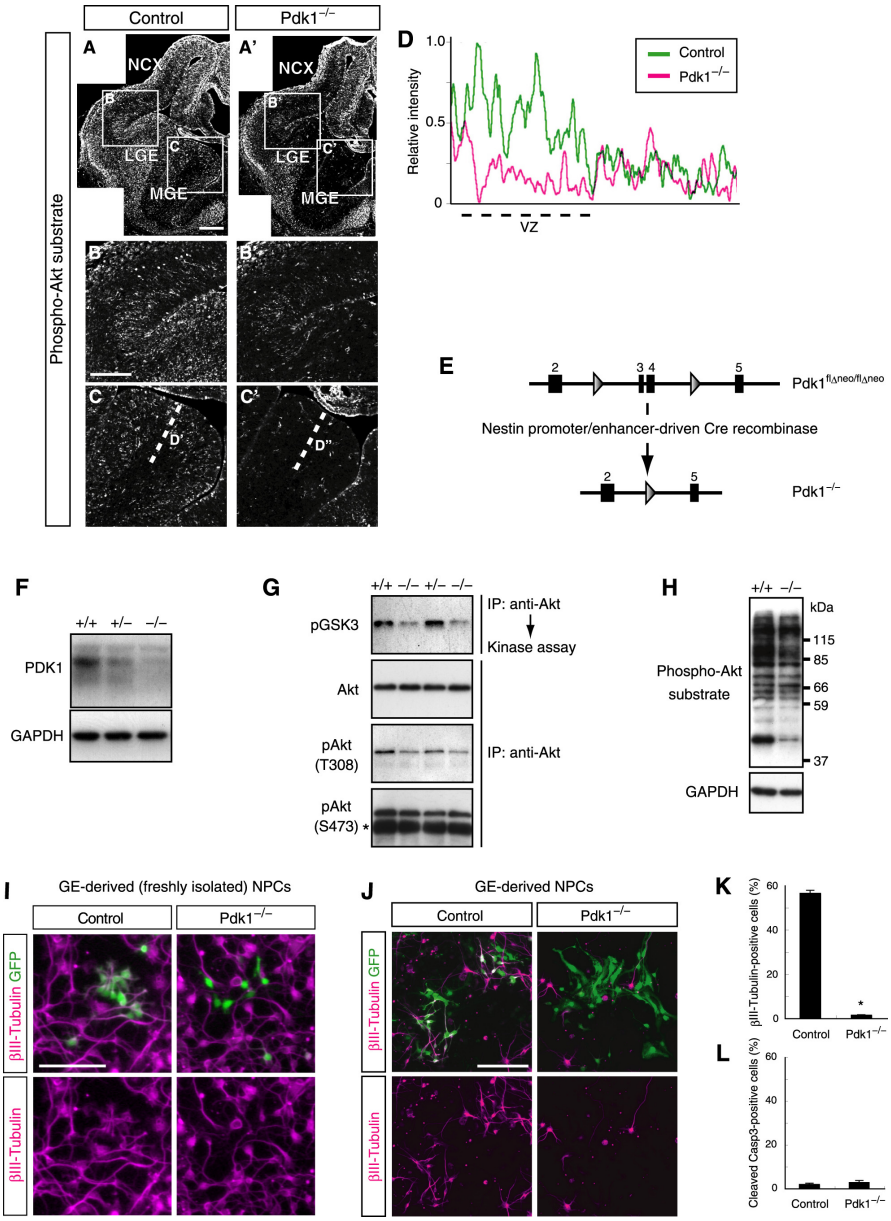


Fig. S1. (*A–D*) Distribution of the phosphorylated form of Akt substrates in the developing brain. Coronal sections of the telencephalon of wild-type control (*A–C*) and *Pdk1*$^{-/-}$ (*A′–C′*) mice at E12.5 were subjected to immunohistochemical analysis with anti–phospho–Akt substrate antibodies. The boxed regions in (*A*) are shown at higher magnification in (*B* and *C*). NCX, neocortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. (*D*) The relative intensity of fluorescence of the dashed lines (*D′* and *D″*) shown in (*C*) was measured using ImageJ software. (*E*) Schematic representation of the *Pdk1* allele containing LoxP sites in introns 2 and 4. Expression of Cre recombinase under the control of the nestin gene promoter/enhancer in mice homozygous for this allele [previously designated *Pdk1*$^{fl\Delta neo/fl\Delta neo}$ mice (10)] yields mice homozygous for disruption of *Pdk1* in the CNS (*Pdk1*$^{-/-}$ mice). Filled boxes and triangles represent *Pdk1* exons and LoxP sites, respectively. (*F*) Immunoblot analysis of PDK1 expression in the forebrain of newborn *Pdk1*$^{-/-}$, *Pdk1*$^{+/-}$, or *Pdk1*$^{+/+}$ mice. The blot was also probed with antibodies to GAPDH as a loading control. (*G*) Akt kinase activity in mouse brain. Immunoprecipitates (IP) were prepared from lysates of mouse forebrain of the indicated *Pdk1* genotypes at P0 using antibodies against Akt. These lysates were assayed for kinase activity with recombinant GSK3 as substrate. Phosphorylated GSK3 was detected by immunoblot analysis (upper panel). Akt immunoprecipitates were also subjected to immunoblot analysis with antibodies to the indicated proteins (lower panels). The asterisk indicates a nonspecific band. It is likely that the abundance of PDK1 was substantially reduced in *Pdk1*$^{-/-}$ mice, given that a 90% decrease in the amount of PDK1 was previously found not to be sufficient for a reduction in the level of Akt phosphorylation on Thr-308 in embryonic tissue (10). (H) Phosphorylation of Akt substrates in NPC culture. NPCs were prepared from 3-day neurosphere cultures of the ganglionic eminences of the indicated *Pdk1* genotypes at E13.5. The cells were lysed and subjected to immunoblot analysis with antibodies to the indicated proteins. (*I–K*) *Pdk1* deletion results in reduced neuronal differentiation. NPCs freshly isolated from the E14.5 medial ganglionic eminence (*I*) or NPCs derived from secondary neurospheres generated from the ganglionic eminences (*J*) were infected with retroviruses encoding GFP for further NPC selection because retroviral infection takes place preferentially in dividing cells. The cells were allowed to differentiate for 2 (*I*) or 3 (*J*) days, and subjected to immunostaining with antibodies to βIII-tubulin and to GFP. The percentage of βIII-tubulin-positive cells among GFP-positive cells in (*J*) was determined (*K*). Data are from 5 different fields. (*L*) Effects of *Pdk1* gene deletion on cell death. NPCs prepared as in (*J*) were allowed to differentiate for 2 days, and subjected to immunostaining with antibodies to cleaved caspase-3 and to GFP. The percentage of cleaved caspase-3-positive cells among GFP-positive cells was determined. Data are from 6 different fields. (Scale bars, 100 μm.)

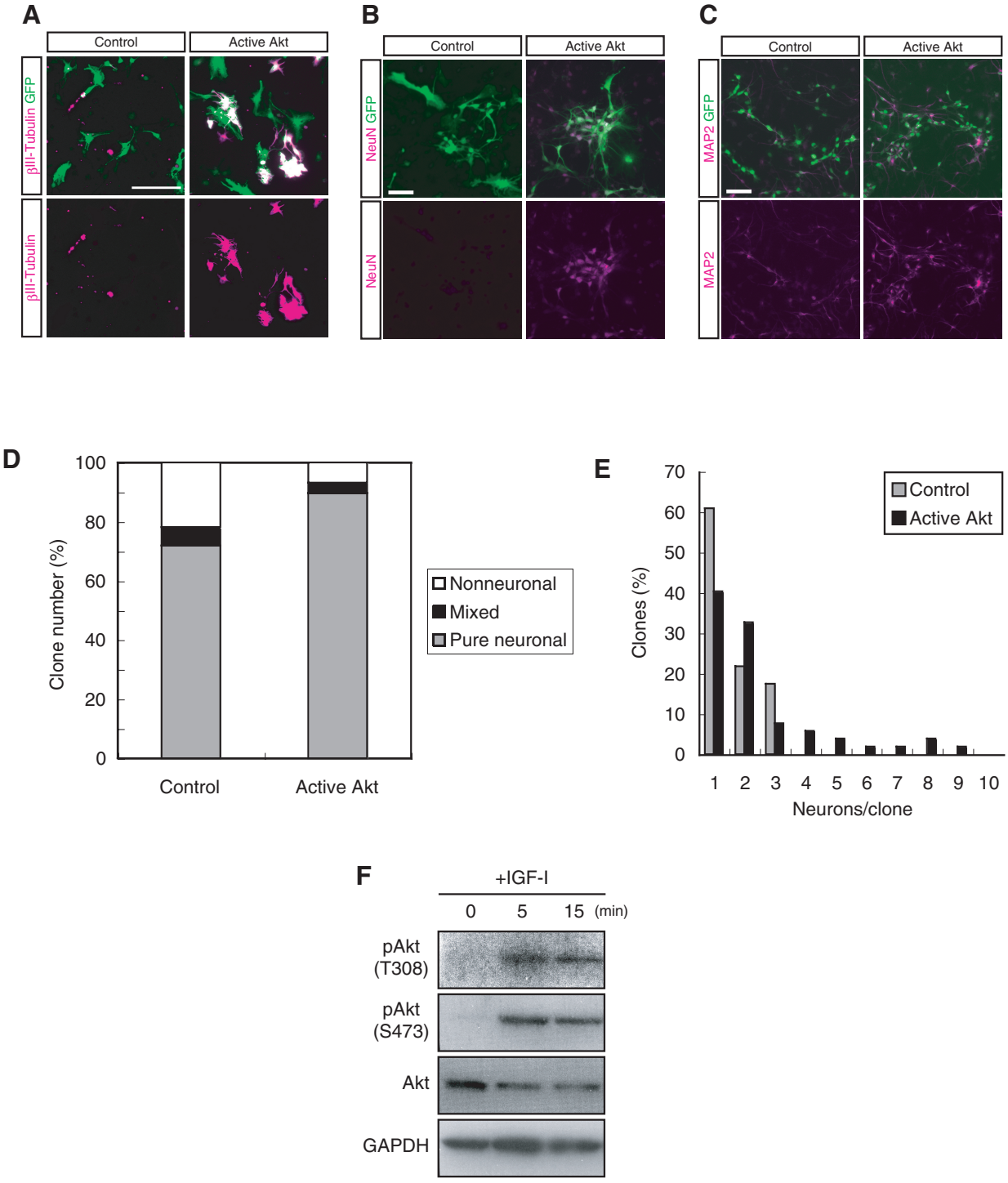


Fig. S2. Role of Akt signaling in neuronal differentiation. (*A*) NPCs prepared from 3-day neurosphere cultures of the E12.5 neocortex were infected with retroviruses encoding GFP alone (Control) or GFP together with an active form of Akt (Active Akt). The infected cells were cultured for 2 days in the absence of FGF-2 and EGF to induce differentiation and then subjected to immunostaining with antibodies to βIII-tubulin and to GFP. (*B* and *C*) NPCs dissociated from 6-day neurosphere cultures were infected with the indicated retroviruses. The infected cells were cultured for 4 days in the absence of FGF-2 and EGF to induce differentiation and then subjected to immunostaining with antibodies to GFP and NeuN (*B*) or MAP2 (*C*). (*D* and *E*) NPCs were prepared from 3-day neurosphere cultures, infected, and analyzed as in Fig. 1*C*, with the exception that infection was performed at a low titer. Each single cell-derived clone was classified according to its fate as neuronal, nonneuronal, or mixed. The proportion of each type of clone was determined (*D*) and the number of cells in each pure neuronal clone was counted (*E*). Data are representative of a total of 3 independent experiments. (*F*) NPCs prepared from 6-day neurosphere cultures were cultured in the absence of FGF-2, EGF, and insulin for 2 h before IGF-1 treatment. The cells were then treated with IGF-1, lysed at the indicated time points, and subjected to immunoblot analysis with antibodies to the indicated proteins. (Scale bars, 100 μm.)

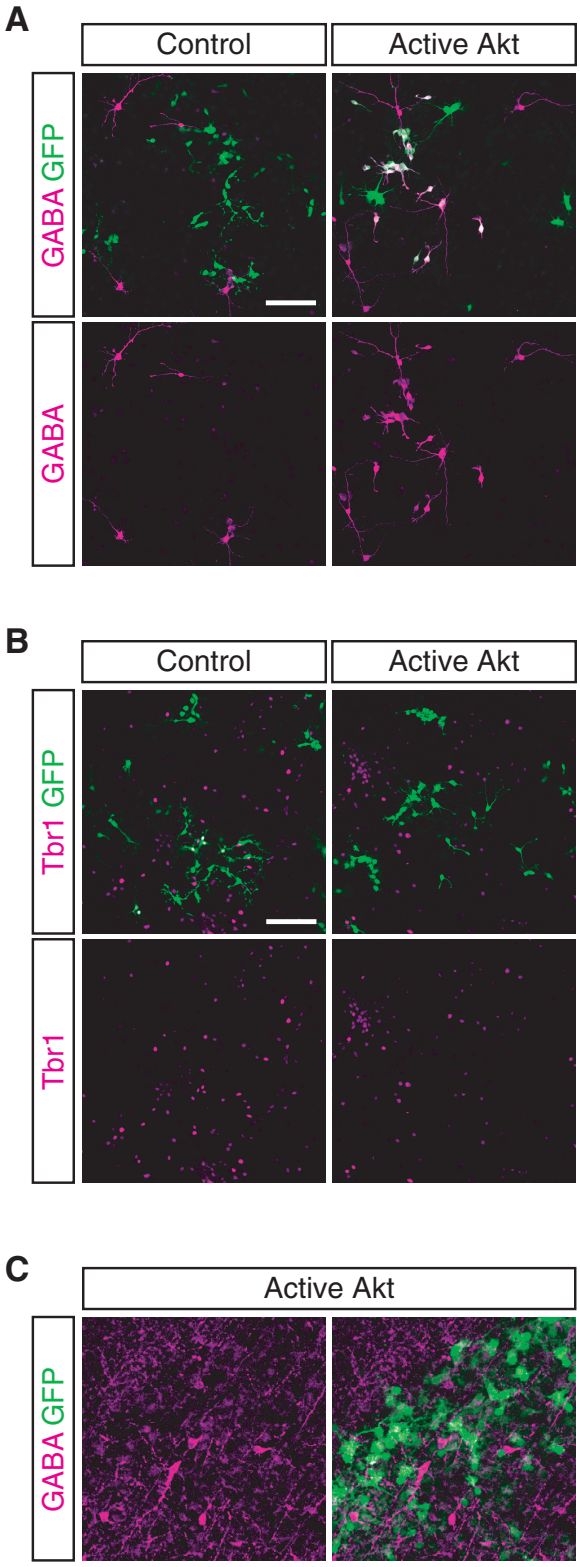


Fig. S3. (*A* and *B*) NPCs prepared from neurosphere cultures generated from the neocortex were infected with the indicated retroviruses and cultured for 3 days in the absence of FGF-2 and EGF to induce differentiation. The cells were then subjected to immunostaining with antibodies to GABA and to GFP (*A*) or to Tbr1 and to GFP (*B*). (*C*) Akt-IRES-GFP plasmid was introduced into the E14.5 VZ of the NCX by in utero electroporation. The fate of the GFP-positive cells was examined at P2 by immunohistochemistry with anti-GFP and anti-GABA. (Scale bars, 100 μm.)

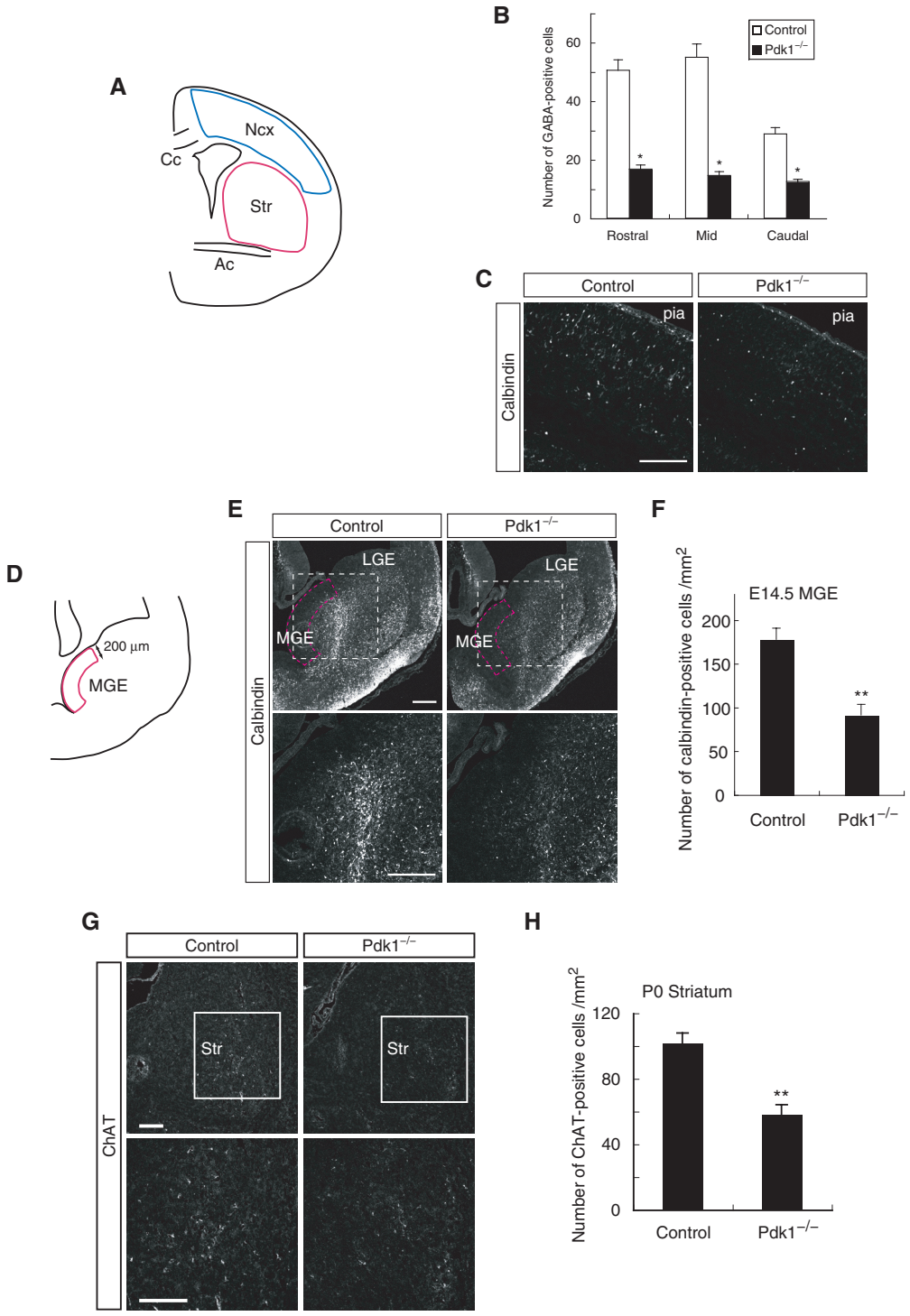


Fig. S4. (*A*) Schematic representation of the brain regions analyzed in Fig. 3 *B* and *E*. Sections that include the corpus callosum (Cc) and anterior commissure (Ac) were analyzed. (*B* and *C*) Brain sections prepared from P0 control or *Pdk1*$^{-/-}$ mice were subjected to immunohistochemistry with antibodies to GABA (*B*) (see also Fig. 3*A*) and to calbindin (*C*). The numbers of GABA-positive cells in 500-μm segments of the neocortex at the indicated levels were determined. Data are from 8 sections of each genotype. *, $P < 0.0001$. (*D*) Schematic representation of the brain regions analyzed in (*E*). A 200-μm area from the ventricular surface of the medial ganglionic eminence was analyzed. (*E* and *F*) Coronal sections at E14.5 were subjected to immunohistochemistry with anti-calbindin. The boxed regions in upper panels are shown at higher magnification in lower panels (*E*). The numbers of calbindin-positive cells per mm^2 in a 200-μm area from the ventricular surface of the medial ganglionic eminence [indicated in (*D*)] were determined (*F*). Data are from 4 different regions. **, $P < 0.005$. (*G* and *H*) Coronal sections of the striatum of mice of the indicated *Pdk1* genotypes at P0 were subjected to immunohistochemistry with antibodies to ChAT. The boxed regions of the upper panels are shown at higher magnification in the lower panels. The number of ChAT-positive cells per mm^2 was determined (*H*). Data are from 4 sections of each genotype. **, $P < 0.005$. (Scale bars, 200 μm.)

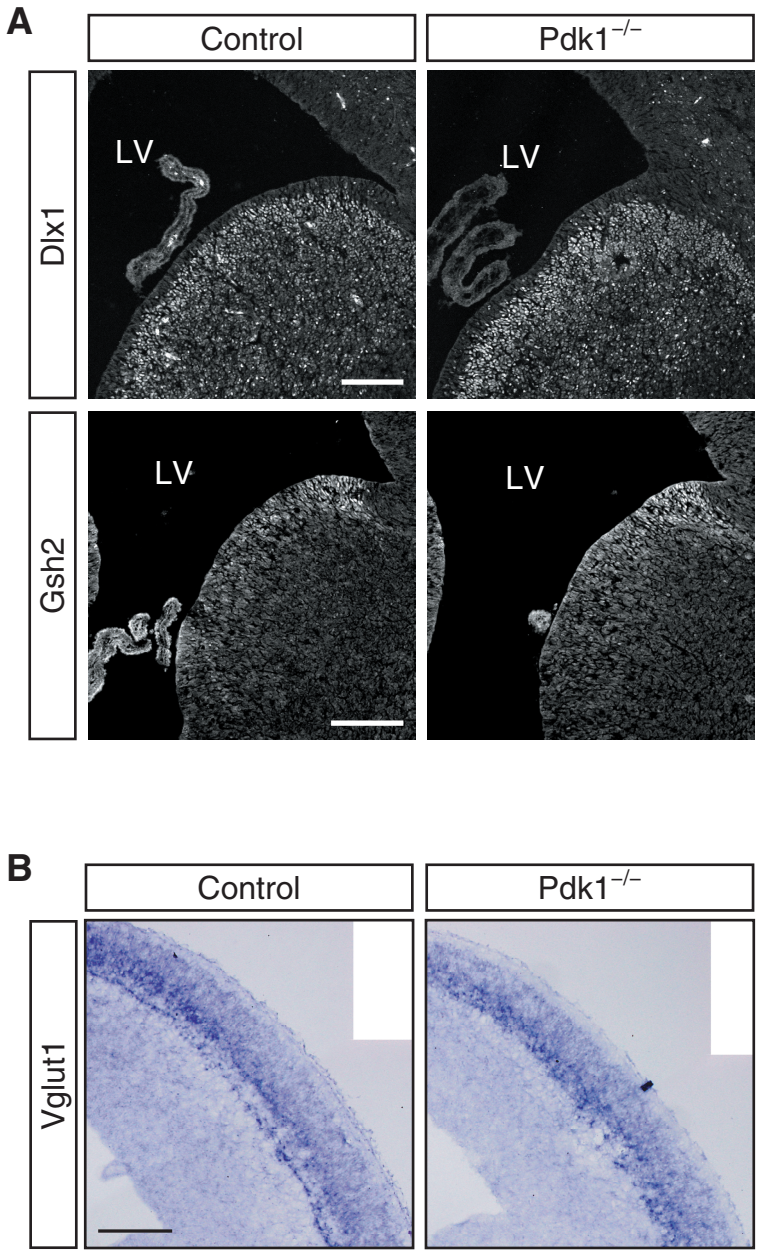


Fig. S5. (*A*) Coronal sections of the lateral ganglionic eminence of mice of the indicated *Pdk1* genotypes at E16.5 (upper panels) or E14.5 (lower panels) were subjected to immunohistochemistry with antibodies to Dlx1 and to Gsh2. LV, lateral ventricle. (*B*) Coronal sections at E16.5 were subjected to in situ hybridization with *Vglut1* probes. (Scale bars, 200 μm.)

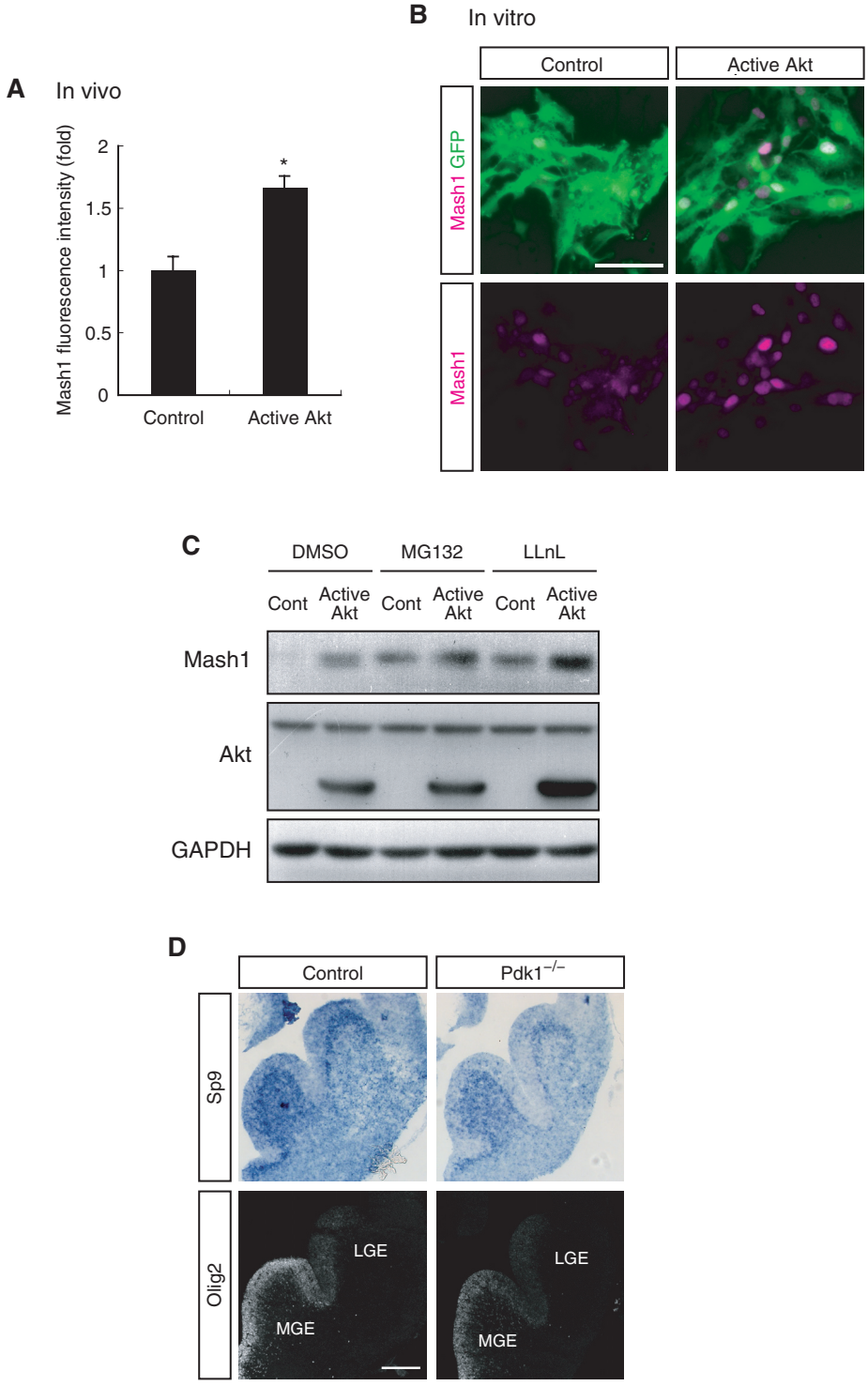
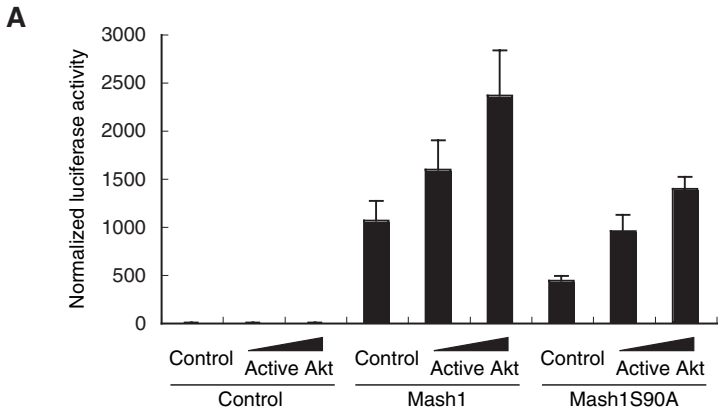
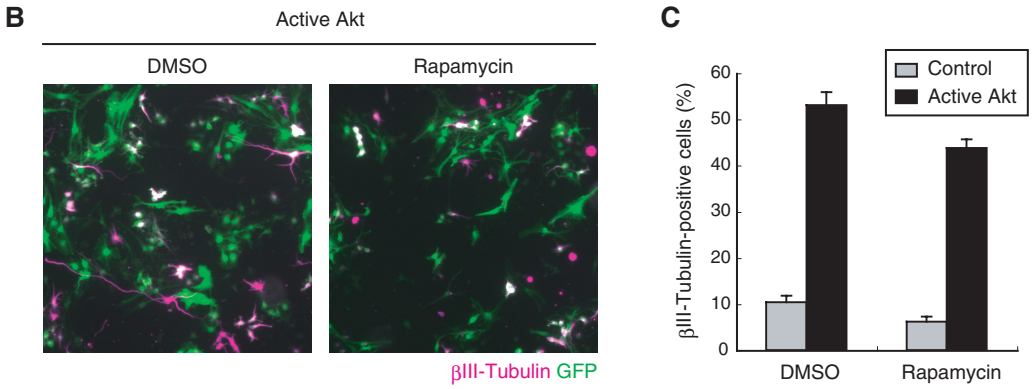


Fig. S6. (*A*) Plasmid vectors encoding GFP alone or GFP with active Akt were injected into the lateral ventricle and introduced into cells at the ventral ventricular zone by electroporation at E11.5. After 2 days, the embryos were fixed and subjected to immunohistochemistry with anti-GFP and anti-Mash1. Fluorescence intensity of Mash1 protein in GFP-positive cells in the medial ganglionic eminence was determined. Data are from at least thirty different cells. *, $P < 0.0001$. (*B*) Expression of Mash1 protein in NPCs. NPCs prepared from 6-day neurosphere cultures were infected with the indicated retroviruses, incubated for 24 h in the absence of FGF-2 and EGF, and subjected to immunocytochemistry with antibodies to the indicated proteins. (*C*) Cos-1 cells were transfected with a Mash1 expression plasmid together with expression vectors for Akt construct as indicated and cultured for 24 h. The cells were then treated with proteasome inhibitors (MG132 or LLnL) for 2 h. The cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins. (*D*) Coronal sections at E13.5 were subjected to in situ hybridization with *Sp9* probes (upper panels) and to immunohistochemistry with antibodies to Olig2 (lower panels). (Scale bars: *B*, 50 μm; *D*, 200 μm.)





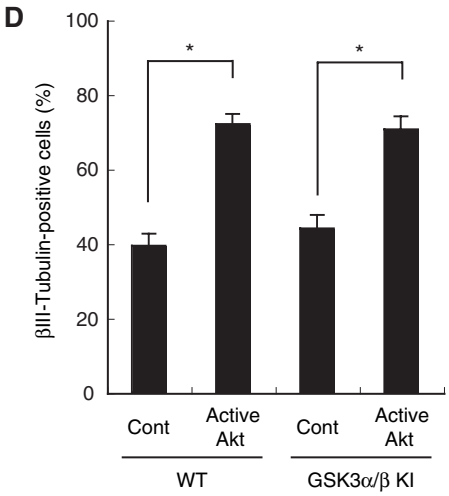


Fig S7. (*A*) NPCs prepared from 6-day neurosphere cultures were transfected with a luciferase reporter plasmid for Mash1 together with expression vectors for wild-type (WT) Mash1 and Mash1S90A, which harbors a mutation on potential Akt sites. The cells were incubated for 24 h, after which the normalized luciferase activity of cell lysates was determined. Data are from 3 independent experiments. (*B* and *C*) Inhibition of the mTOR pathway did not cancel Akt-induced neuronal differentiation. NPCs prepared from 6-day neurosphere cultures were infected with the indicated retroviruses. The infected cells were cultured for 3 days in the absence of FGF-2 and EGF to induce differentiation together with the treatment of DMSO or Rapamycin (10 nM). The cells were then subjected to immunostaining with antibodies to βIII-tubulin and to GFP (*B*). The percentage of βIII-tubulin-positive cells among GFP-positive cells was determined (*C*). Data are from 4 independent experiments. (*D*) Role of GSK3 in regulation of neuronal differentiation. NPCs dissociated from 6-day neurosphere cultures prepared from WT mice and the knock-in mice (GSK3α/β KI, GSK3$\alpha^{+/S21A}$;GSK3$\beta^{+/S9A}$) were infected with the indicated retroviruses. The infected cells were cultured for 3 days in a differentiation condition. The percentage of bIII-tubulin-positive cells among GFP-positive cells was determined. Data are from five different fields. *, $P < 0.0001$.

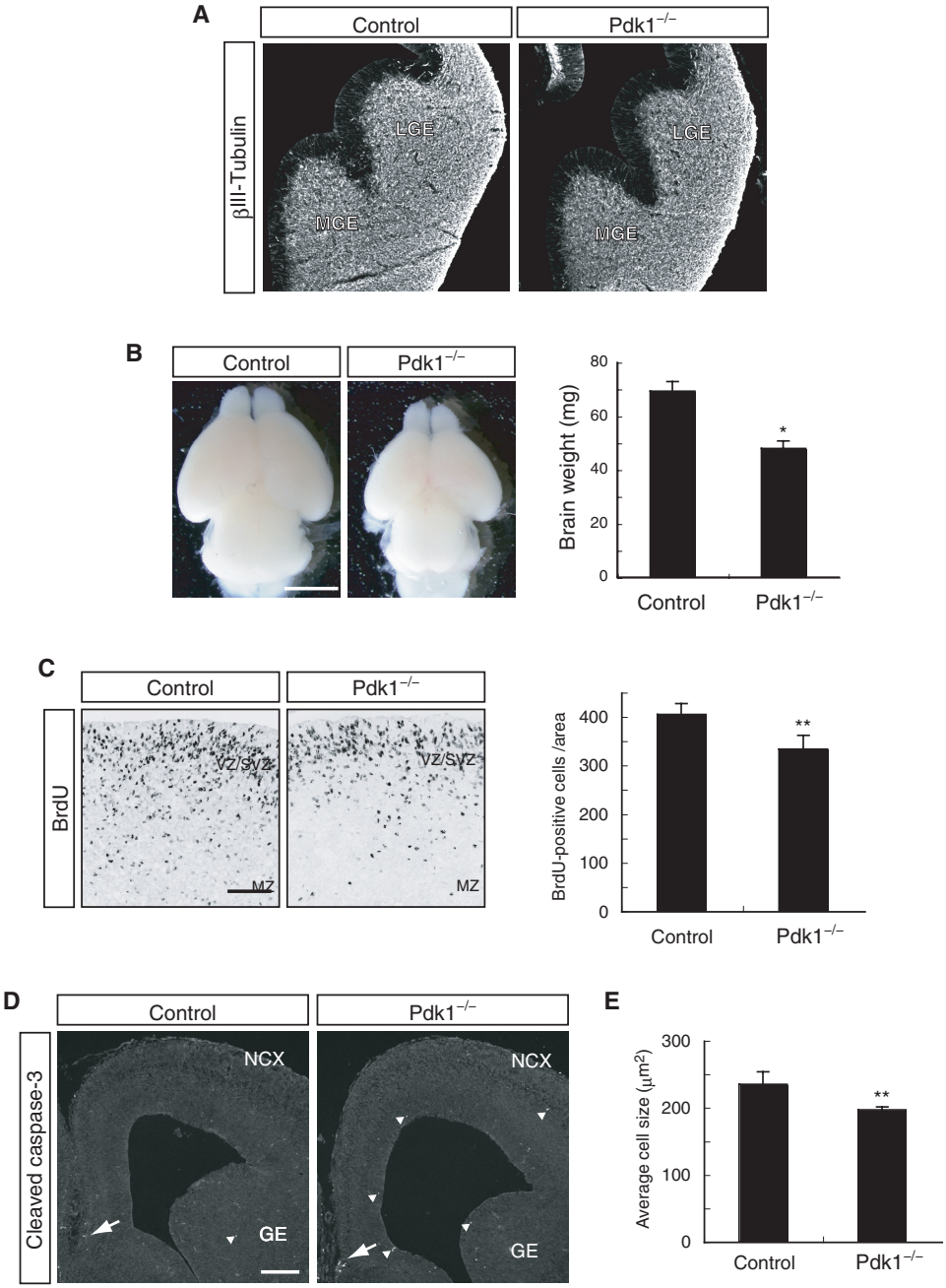


Fig. S8. (*A*) Coronal sections of the telencephalon of control and *Pdk1*$^{-/-}$ mice at E13.5 were subjected to immunohistochemical analysis with an antibody to βIII-tubulin. (*B–E*) PDK1 regulates proliferation, survival and size of NPCs. (*B*) Dorsal view of the brain of newborn (P0) control and *Pdk1*$^{-/-}$ mice (left panels). Weights of the brain (including olfactory bulb, telencephalon, diencephalon, and mesencephalon) of newborn control and *Pdk1*$^{-/-}$ mice (right panel) are indicated. Data are means ± SD of values from 5 animals of each genotype. (*C*) Proliferation of NPCs in the VZ and SVZ of control and *Pdk1*$^{-/-}$ mice. The brain of embryos was pulse-labeled with BrdU (see *SI Methods*) in utero at E16.5, and coronal sections of the brain were then subjected to immunohistochemistry with antibodies to BrdU (left panels). VZ, ventricular zone; SVZ, subventricular zone; MZ, mantle zone. The number of BrdU-positive cells per 0.15 mm^2 in the VZ and SVZ of the ganglionic eminence was counted (right panel). Data are means ± SD of values from 4 sections of each genotype. Note that the number of BrdU-positive cells in the VZ and SVZ was reduced by approximately 18% in the mutant brain compared with that in the control brain at E16.5. These results indicated that PDK1 is required for efficient proliferation of NPCs in the developing brain. (*D*) Immunohistochemical analysis of the distribution of cleaved caspase-3 in the developing telencephalon at E14.5. An increased level of apoptosis (arrowheads) was apparent in the brain of *Pdk1*$^{-/-}$ mice, including around the fornix, where frequent apoptosis was observed in WT mice (arrows). It is, however, difficult to quantify the level of cell death in the developing brain, because of the rapid clearance of cell corpses after apoptosis (11). (*E*) Cell size in the brain of control and *Pdk1*$^{-/-}$ mice. The average cell size was estimated by counting cell number per 0.18 mm^2 in the striatum at E18.5. Data are means ± SD of values from 4 sections of each genotype. Note that the average cell size estimated from cell number per area in the striatum of E18.5 animals was reduced by approximately 16% in the mutant mice compared with controls. *, $P < 0.0001$; **, $P < 0.01$. The reduced extent of cell proliferation, increased level of cell death, and smaller cell size apparent in the brain of *Pdk1*$^{-/-}$ mice thus likely account, at least in part, for the small-brain phenotype of these animals. (Scale bars: *B*, 2 mm; *C*, 100 μm; *D*, 200 μm.)