Selective induction of neocortical GABAergic neurons by the PDK1-Akt pathway through activation of Mash1

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Extracellular stimuli regulate neuronal differentiation and subtype specification during brain development, although the intracellular signaling pathways that mediate these processes remain largely unclear. We now show that the PDK1-Akt pathway regulates differentiation of telencephalic neural precursor cells (NPCs). Active Akt promotes differentiation of NPC into γ-aminobutyric acid-containing (GABAergic) but not glutamatergic neurons. Disruption of the Pdk1 gene or expression of dominant-negative forms of Akt suppresses insulin-like growth factor (IGF)-1 enhancement of NPC differentiation into neurons in vitro and production of neocortical GABAergic neurons in vivo. Furthermore, active Akt increased the protein levels and transactivation activity of Mash1, a proneural basic helix-loop-helix protein required for the generation of neocortical GABAergic neurons, and Mash1 was required for Akt-induced neuronal differentiation. These results have unveiled an unexpected role of the PDK1-Akt pathway: a key mediator of extracellular signals regulating the production of neocortical GABAergic neurons.

GABAergic neuronal differentiation | neural precursor cells | telencephalon

The mammalian central nervous system (CNS) arises from common precursor cells (neural precursor cells, or NPCs), which proliferate and generate both neurons and glia (1, 2). The development of the CNS involves sequential waves of neurogenesis and gliogenesis (3–5) and requires an appropriate balance between the proliferation and differentiation of NPCs and their progeny (6). Accumulating evidence has shown that extrinsic cues, including cell-cell interactions and secreted molecules, are key determinants of NPC fate. For instance, extrinsic factors such as Wnt-7a, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-1, bone morphogenetic protein (BMP), erythropoietin, and the neurotrophic factors brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and neurotrophin-3 promote neuronal differentiation by several mechanisms, including the selective expansion of neuronal progenitors, enhancement of the survival of neurons (or their progenitors), and favoring neuronal fate (7–10). However, few studies have investigated the underlying mechanisms that mediate the neurogenic effects of these extrinsic factors.

Diverse types of neurons are produced during CNS development. Two major types of neurons arise during development of telencephalon: excitatory, glutamatergic neurons in the dorsal region, and inhibitory, GABAergic neurons in the ventral region (11, 12). A key issue in developmental neurobiology concerns the mechanisms responsible for determining the generic and specific characteristics of neurons. These mechanisms are mediated by combinations of transcription factors including proneural basic helix-loop-helix (bHLH) and homeodomain proteins (10, 13, 14). The bHLH proteins neurogenin1 (Ngn1), Ngn2, and Mash1 are important for neuronal fate determination of undifferentiated NPCs (15–17). Ngn and Mash1 are expressed in complementary patterns in the dorsal and ventral parts of the telencephalon, contributing to the generation of glutamatergic and GABAergic neurons, respectively (17, 18). Although some transcriptional/posttranscriptional regulations on these transcription factors have been revealed, it largely remains unclear how they are regulated by neurogenic extrinsic cues during CNS development (7, 19–24).

A signaling module comprised of phosphoinositide-dependent kinase 1 (PDK1) and Akt is activated via phosphoinositide 3-kinase (PI3K) by various extrinsic cues. This signaling module plays a pivotal role in the regulation of cell survival, proliferation, and size in many systems (25–28). In fact, a number of studies has suggested the roles of the PI3K-Akt pathway in NPC proliferation and/or differentiation (29–33). However, even though PDK1 and all 3 members of the Akt family are expressed in the developing CNS, genetic evidence for their importance in CNS development is lacking. Mice deficient in 1 or 2 Akt family members exhibit little embryonic defects in the CNS, possibly because of functional compensation among the family members (28, 34, 35). Pdk1 knockout mice, in which Akt activity is almost completely absent, die before CNS development starts (26). We therefore generated mice in which Pdk1 is conditionally ablated in the CNS to examine the role of the PDK1-Akt pathway in CNS development.

Here we show that PDK1 is essential for regulation of NPC differentiation during CNS development. Disruption of Pdk1 or expression of dominant-negative forms of Akt reduced neuronal differentiation of NPCs. Moreover, constitutively active Akt promoted NPC differentiation into GABAergic but not glutamatergic neurons. Consistently, CNS-specific ablation of Pdk1 impaired production of GABAergic but not glutamatergic neurons in the telencephalon. We further show that Akt activates Mash1, a transcription factor important for the differentiation of a subset of GABAergic neurons, and that Akt has little effect on neuronal differentiation in Mash1-deficient NPCs. Together, these results demonstrate that the PDK1-Akt pathway regulates, through activation of Mash1, neuronal differentiation and subtype specification during telencephalic development.

Results

Activation of Akt in NPCs of the Developing Telencephalon. We first examined where the PDK1-Akt pathway is activated in the developing brain by immunohistochemical analysis with antibodies that react specifically with the phosphorylated form of Akt substrates (anti-phospho-Akt substrate antibodies). In the developing telencephalon, immunoreactivity was detected in the mantle of the neuroepithelium (anti-phospho-Akt substrate antibodies). In the developing telencephalon, immunoreactivity was detected in the mantle of the neuroepithelium.
telencephalon [supporting information (S1 Fig. S1 A–D)]. While staining of the mantle was not affected, staining of the VZ and SVZ was greatly reduced in mice having a CNS-specific disruption of Pdk1 (Fig. S1 A–D) (see below). This confirms antibody specificity, and shows that PDK1 is active in the VZ and SVZ, where proliferating NPCs reside.

Reduced Potential of Pdk1<sup>−/−</sup> NPCs to Differentiate into Neurons. We then examined the in vivo role of PDK1 during brain development by generating mice in which Pdk1 is conditionally ablated in the CNS (Fig. S1E). Compared with control (Pdk1<sub>flox</sub>+/−) mice, the amount of PDK1 in forebrain extracts was markedly reduced in heterozygous mutant mice (Pdk1<sub>flox</sub>+/−, harboring the nestin enhancer-Cre transgene), and the protein was virtually undetectable in homozygous mutant mice (Pdk1<sub>flox</sub>/− mice harboring the transgene), at postnatal day 0 (P0) (Fig. S1F). For the purposes of this study, these 3 types of mice will be referred to hereafter as Pdk1<sub>flox</sub>/−, Pdk1<sub>flox</sub>+/−, and Pdk1<sub>flox</sub>+/−, respectively. The in vitro kinase activity of Akt immunoprecipitates prepared from brain extracts of Pdk1<sup>−/−</sup> mouse was reduced by 60% compared with that in wild-type or heterozygous mutant mice, and this reduction in Akt activity was accompanied by reduced phosphorylation of Akt on Thr-308, which is catalyzed by PDK1 (Fig. S1G). In contrast, phosphorylation of Akt on Ser-473, which is catalyzed by TORC2, was unaffected (Fig. S1G). The intensity of the bands detected by anti-phospho-Akt substrate in NPC culture extracts of Pdk1<sup>−/−</sup> mice was also lower than that in the corresponding wild-type extracts (Fig. S1H). These results suggested that the abundance and activity of PDK1 was substantially reduced in the brains of Pdk1<sup>−/−</sup> mice.

Given that PDK1 is active in NPCs, we next investigated the differentiation potential of NPCs obtained from Pdk1<sup>−/−</sup> mice. NPCs freshly isolated from the ganglionic eminence (GE) as well as GE-derived NPC aggregates (so-called neurospheres) prepared from floating culture were differentiated. The numbers of neurons produced from Pdk1<sup>−/−</sup> NPCs were decreased in both cultures (Fig. L4 and Fig. S1 I–K). Since the percentage of dying cells was small (<3%) even in the absence of Pdk1 gene under the condition used (Fig. 1B and Fig. S1L), the reduction of neuronal number by Pdk1 gene deletion was unlikely to be due to an increased cell death. These results suggest that inhibition of PDK1 signaling reduces the potential of NPCs to differentiate into neurons.

**Akt Promotes Neuronal Differentiation of Telencephalic NPCs In Vitro.**

We next examined whether Akt regulates neuronal differentiation of NPCs. Infection of neocortex (NCX)-derived NPCs with a retrovirus encoding a constitutively active form of Akt markedly increased the proportion of cells that express the neuronal markers βIII-tubulin, MAP2, and NeuN (Fig. 1C and Fig. S2 A–C). Conversely, dominant-negative forms of Akt (Akt3A or AktKA) reduced the proportion of βIII-tubulin-positive cells (Fig. 1D). These results suggest a pivotal role for Akt in the neuronal differentiation of embryonic NPCs.

At least 2 possibilities might account for the increase in the proportion of neurons induced by Akt activation: Akt might instruct commitment of undifferentiated NPCs to neuronal fate, or it might selectively promote the proliferation of committed neuronal progenitors. To distinguish between these possibilities, we performed an in vitro clonal assay by infecting NPCs with retroviruses at a low titer and classifying each single-cell-derived clone on the basis of its fate as a neuronal clone, nonneuronal clone, or mixed clone. This method enabled us to examine neuronal commitment directly, given that the extent of cell death in our culture system was negligible (3%, revealed by immunocytochemical analysis with antibody to cleaved caspase-3) (7). Expression of activated Akt increased the percentage of pure neuronal clones (Fig. S2D), suggesting that Akt promotes neuronal commitment of NPCs. The number of neurons in the pure neuronal clones was also increased (Fig. S2E), suggesting that Akt also promotes the proliferation of neuron-producing progenitor cells. Together, these results indicate that Akt affects neuronal fate and the proliferation of neuronal progenitor cells simultaneously, resulting in a large increase in the size of the neuronal population.

IGF-1 is expressed in the embryonic telencephalon (36) and has been shown to enhance neuronal differentiation in NPC cultures (8). Since IGF-1 is a well-known activator of the PDK1-Akt pathway, we asked whether this pathway mediates the effects of IGF-1 on neuronal differentiation. IGF-1 treatment indeed increased the proportion of βIII-tubulin-positive cells among GFP<sup>+</sup> cells (Fig. S2F). The amounts of βIII-tubulin and cells among GFP<sup>+</sup> cells was determined. Data from 3 independent experiments. (C) NPCs prepared from 3-day neurosphere cultures of the E12.5 NCX were infected with pCAGGS-floxed-GFP (Active Akt) retroviruses, and differentiated for 2 days. The percentage of βIII-tubulin<sup>+</sup> cells among GFP<sup>+</sup> cells was determined. Data from 4 independent experiments. (D) Primary neuroepithelial cells prepared from the NCX were infected with the indicated retroviruses. The cells were further cultured for 3 days in suspension and then induced to differentiate and analyzed as in (C). (E) NPCs prepared from 6-day neurosphere cultures of the NCX were infected with the indicated retroviruses and cultured for 2 days in the absence of FGF-2, EGF and insulin, and either the absence or presence of IGF-1. The cell lysates were then immunoblotted with antibodies to the indicated proteins. Asterisks indicate a statistical difference between experimental groups (*, P < 0.0001; **, P < 0.03).

**Selective Induction of GABAergic Neurons by Akt In Vitro.**

Most NPCs residing on the dorsal side of the telencephalon give rise to excitatory, glutamatergic neurons, whereas those on the ventral side produce inhibitory, GABAergic neurons (11, 12). Several reports, however, have shown that cultured NPCs derived from the NCX have ventralized characteristics with marker expression patterns intermediate between the NCX and GEs (cf. up-regulation of Mash1 in Fig. 1E) and generate both glutamatergic and GABAergic neurons (37–39). NCX-derived NPCs, which are prepared as NCX-derived NPCs (<IGF-1–IGF-I> or Akt–Akt<sub>KA</sub>) and differentiated for 2 days in the absence of FGF-2, EGF and insulin, and either the absence or presence of IGF-1. The cell lysates were then immunoblotted with antibodies to the indicated proteins. Asterisks indicate a statistical difference between experimental groups (*, P < 0.0001; **, P < 0.03).
Akt Promotes Production of GABAergic Neurons in the Developing GEIs. Given that Akt promotes GABAergic neuronal differentiation in vitro, we next asked whether forced expression of active Akt might affect GABAergic neuronal differentiation in the developing GEIs in utero. Expression plasmids were injected into the lateral ventricles of mouse embryos at E12.5, and were introduced into NPCs in the VZ of the GEIs by electroporation. Electroporation of plasmids encoding active Akt and GFP, but not control plasmid encoding GFP alone, markedly increased the number of cells strongly positive for GABA, as determined by immunohistochemical analysis (Fig. 2 H and I). In contrast, forced expression of active Akt in the NCX by in utero electroporation did not induce ectopic differentiation of GABAergic neurons (Fig. S3C). This suggests that Akt on its own is not sufficient to convert NPC fate from glutamatergic to GABAergic, and that a target or cofactor of Akt might be missing in the NCX for inducing GABAergic neurons.

We further tested whether inhibition of Akt might affect the differentiation of GABAergic neurons. To test this, a dominant-negative form of Akt was introduced into NPCs at the medial ganglionic eminence (MGE) by electroporation in the telencephalic explant culture. We found that the number of the cells positive for calbindin (a GABAergic neuron marker) was greatly reduced by expression of dominant-negative Akt (Fig. 2J). These observations support the notion that the Akt pathway promotes NPC differentiation into GABAergic neurons in vivo. Importantly, these in utero experiments, in addition to in vitro results, suggest that Akt regulates NPC differentiation in a cell-autonomous manner.

Reduced Number of GABAergic, But Not Glutamatergic, Neurons in the NCX of Pdk1−/− Mice. Given that Akt promoted the differentiation of GABAergic but not glutamatergic neurons in vitro, we investigated whether disruption of Pdk1 selectively affected the generation of GABAergic neurons in the developing telencephalon by immunohistochemistry using antibodies to neuronal subtype-specific markers. Subpopulations of GABAergic neurons generated in the ventral telencephalon migrate to the NCX, olfactory bulb, and striatum (11, 12), and a large proportion of neocortical interneurons are produced in a Mash1-dependent manner (16). The number of GABA-positive neurons in the NCX was markedly decreased in Pdk1−/− mice compared with wild-type mice at all rostrocaudal levels analyzed (Fig. 3 A and B and Fig. S4 A and B). The number of calbindin-positive neurons was also reduced in the NCX of Pdk1−/− mice (Fig. S4C).

We further examined differentiation of GABAergic neurons in Pdk1−/− mice at earlier stages. We found that Pdk1 deletion substantially reduced expression levels of Lhx6 at E13.5, a marker for interneurons derived from the MGE (Fig. 3C) as well as the number of calbindin-positive cells in the MGE at E14.5 (Fig. S4 D–F). These results indicate that PDK1 already affects the production of GABAergic neurons, before their migration to the NCX (see also Fig. 4E–G and Fig. S6D for early phenotypes of Pdk1−/− mice). We also examined whether striatal neurons are affected by Pdk1 ablation. Staining of brain sections from newborn (P0) mice with antibodies against neuropeptide Y (NPY) or choline acetyltransferase (ChAT), markers of subsets of inhibitory striatal interneurons, revealed that both types of interneurons were greatly reduced in Pdk1−/− mice compared with control animals (Fig. 3D and E and Fig. S4 G and H). In contrast, the overall proportion of striatal cells positive for DARPP32, a D1 receptor-associated protein found in striatal projection neurons, was not markedly affected by Pdk1 deletion (Fig. 3F). Consistently, the expression patterns of Dbx1 and Gsh2, regional markers of the lateral ganglionic eminence (LGE) (which gives rise to striatal and olfactory GABAergic neurons), were unaffected by Pdk1 deletion (Fig. S5A). These results together

and active Akt did not significantly increase the amount of Vglut1 protein relative to that of GAPDH protein in these cells. In addition, reverse transcription (RT) and real-time polymerase chain reaction (PCR) analysis revealed that expression of active but not inactive Akt increased the amount of mRNA for Gad65 (another GABAergic neuronal marker) but slightly reduced that of Vglut1 mRNA (Fig. 2 C and D). Furthermore, the proportion of GABA-positive but not Tbr1 (a marker of the glutamatergic lineage)-positive neurons was greatly increased by expression of active Akt (Fig. 2 F and G and Fig. S3 A and B). Since GABAergic neurons are normally produced from the GEIs, we also confirmed that the proportion of GABA-positive neurons, the levels of Gad65 mRNA and GAD67 protein were significantly increased when active Akt was expressed in GE-derived NPCs (Figs. 2 B and E and 5C). Together, these results suggest that Akt selectively promotes the generation of GABAergic neurons from NPCs.
suggested that PDK1 is essential for the generation of some, but not all, GABAergic neurons.

In contrast to interneurons, cortical projection neurons were largely unaffected by disruption of Pdk1. Expression of the glutamatergic neuron markers Vglu1 and Tbr1 was relatively normal (Fig. 3 G and H and Fig. S5B). These results suggest that PDK1 is important for producing interneurons but not projection neurons in the cortex.

**Akt Increases the Transcription Activity and Protein Levels of Mash1.**

To address the mechanism by which the Akt pathway promotes the differentiation of GABAergic neurons, we focused on regulation of Mash1, a proneural bHLH protein that is required for the production of a subpopulation of GABAergic neurons in the NCX and striatum (ie, loss of Mash1 dramatically reduced GABAergic neocortical interneurons and striatal interneurons including NPY- and ChAT-positive cells but had little effect on striatal projection neurons) (16, 40). NCX-derived NPCs were transfected with vectors for Akt constructs, together with a vector encoding Mash1 and a Mash1-reporter gene construct containing the luciferase gene under the control of Mash1 binding sites (41). Expression of active Akt, but not that of Akt3A, increased Mash1-dependent transcriptional activity in a concentration-dependent manner (Fig. 4A).

Whereas active Akt increased the activity of ectopic Mash1 in NPCs (Fig. 4A), it did not increase the amounts of endogenous Mash1 mRNA in NPCs (Fig. 4B), suggesting that Akt modulates Mash1 activity at a post-transcriptional level. Indeed, expression of active Akt increased the amount of endogenous Mash1 protein in NPCs as judged by both immunoblot analysis (Fig. 4C) and immunostaining both in vitro and in vivo (Fig. S6A and B). Furthermore, expression of active Akt markedly suppressed degradation of Mash1 protein in the presence of the protein synthesis inhibitor cycloheximide in Cos-1 cells (Fig. 4D). We also observed that active
Akt did not markedly increase the amount of Mash1 protein in the presence of proteasome inhibitors (Fig. S6C). These results together indicate that Akt increases the amounts of Mash1 protein by increasing its stability.

As mentioned above, we found that IGF-1 treatment promotes neuronal differentiation through Akt activation. We also found that IGF-1 treatment of the NPC culture increased the amount of Mash1 protein, and that this was in part suppressed by the expression of dominant-negative forms of Akt (Fig. 1E). Thus, Akt may regulate the mediation of Mash1 protein abundance by extrinsic neurotransmitter factors.

We next examined the abundance of Mash1 in the developing Pdk1−/− mouse brain. The amounts of Mash1 in the MGEs of Pdk1−/− mice were substantially reduced compared with those of the controls as judged by both immunoblot analysis and immunohistochemistry (Fig. 4E–G). We further found that Pdk1 deletion reduced expression levels of Mash1-dependent genes Sp9 and Olig2 (Fig. S6D) (42). These results confirm that PDK1-Akt signaling regulates the abundance and activity of Mash1 in vivo.

Requirement of Mash1 for Akt-Induced Neuronal Differentiation.

Given that Akt activated Mash1 in NPCs, we next examined whether Mash1 is necessary for Akt-induced neuronal differentiation. NCX- or GE-derived NPCs prepared from mice that were either heterozygous or homozygous for Mash1 deletion (43) were infected with retroviruses encoding GFP alone or GFP together with active Akt. Whereas expression of active Akt markedly promoted neuronal differentiation of the Mash1+/+ or Mash1−/− NPCs as judged by the expression of jIII-tubulin, it had little effect on that of the Mash1−/− NPCs as revealed by immunocytochemistry (Fig. 5A) and immunoblot analysis (Fig. 5B). We also observed that expression of active Akt significantly increased the amounts of the GABAergic marker GAD67 in the Mash1+/+ NPCs, but not in the Mash1−/− NPCs derived from GE (Fig. 5C).

These results demonstrate that Mash1 is required for Akt-mediated GABAergic neuronal differentiation.

Discussion

The PDK1-Akt pathway contributes to regulation of the survival, proliferation, size, or metabolism of various cell types (27), but the precise role of this pathway in neural development has not been well understood (28). We have now provided several lines of evidence demonstrating a previously unrecognized role of this pathway in the promotion of neuronal differentiation, in particular that of a subset of GABAergic neurons, during mouse telencephalic development. First, disruption of Pdk1 resulted in the loss both of the neurogenic potential of NPCs in vitro and of a subset of GABAergic neurons in vivo. Second, ectopic expression of an active form of Akt promoted the production of (GABAergic) neurons in vitro and in vivo, whereas expression of dominant-negative forms of Akt inhibited this production, presumably in a cell-autonomous manner. Third, Akt was found to activate the bHLH protein Mash1, a key regulator of differentiation of GABAergic neurons (16, 17, 40). Akt signaling was highly active in the Mash1-positive NPCs localized in the VZ and SVZ of the MGE and LGE, which generate GABAergic neurons, supporting an in vivo role of PDK1-Akt signaling in the generation of such neurons. As development proceeds, this signal may also act in migrating GABAergic interneurons since PI3K has been reported to regulate tangential migration of neocortical interneurons (44).

Proneural bHLH proteins are key determinants of neuronal fate in the developing telencephalon (13, 15), and several studies have shown how these proteins are regulated posttranscriptionally by extrinsic cues (20–24). Our data now suggest that the PDK1-Akt pathway mediates regulation of Mash1 by extrinsic cues. Candidates for such extrinsic cues that activate the PDK1-Akt pathway during telencephalic development include secreted factors that are capable both of promoting differentiation of GABAergic neurons and of activating PDK1-Akt signaling, including IGF-1, Shh, GDNF, and BDNF (9). Other neurogenic factors such as erythropoietin, PDGF, and EGF are also potential candidates. Indeed, we found that IGF-1 promoted neuronal differentiation and increased Mash1 protein in our culture system in an Akt-dependent manner. Furthermore, any endogenous signals that induce Akt activation in the ventral telencephalon may control the timing and extent of the generation of GABAergic neurons.

Extensive neuronal diversity is central to the many complex functions of the CNS. The PDK1-Akt pathway appears to contribute to this diversity in the telencephalon, given that activation of PDK1-Akt signaling induced expression of the GABAergic neuronal markers GAD65, GAD67, and GABA but not that of the glutamatergic neuronal markers VGLUT1 and Tbr1. This selective action of the PDK1-Akt pathway in neuronal differentiation may depend on the selective requirement of Mash1 among proneural bHLH proteins for this pathway. The mechanism by which Akt activates Mash1 appears to involve the stabilization of Mash1 protein, but the direct target of Akt in this process remains to be determined. Although we detected a consensus sequence for Akt phosphorylation at Ser-90 of the rat Mash1 (RQRSSS), mutation of this residue to alanine did not affect Akt-induced activation of Mash1 (Fig. S7A). We found that 2 well-established downstream pathways of Akt, mTOR and GSK3, were not involved in Akt-induced neuronal differentiation (Fig. S7 B–D). PDK1 is also known to activate other downstream targets than Akt, including p70 S6 kinase and p90 Rsk, which might contribute, at least partly, to the Pdk1−/− phenotypes (25).

Pdk1 mutant mice did not exhibit a severe loss of MGE observed in Mash1 mutant mice (Fig. S8A) (16). We think that this difference might be ascribable to at least 2 possibilities, based on our findings that PDK1 deletion reduced Mash1 protein, but did not eliminate it. One possibility is that high protein levels of Mash1 are necessary
for GABAergic neurogenesis but not for the maintenance of MGE cells, and PDK1 regulates only the former function of Mash1. The other possibility is that PDK1 regulates Mash1 qualitatively, such as the target specificity. In either case, although our results are consistent with the notion that the PDK1-Akt pathway promotes GABAergic neurogenesis, the increase in size of Mash1 protein, in a small number of the Mash1-1-dependent function for PDK1 in NPC population.

Recent studies have provided convergent evidence for impairment of Akt1 signaling in schizophrenia (45). A reduced abundance of Akt1 in the brain was found to be significantly associated with schizophrenia, and Akt1 deficiency conferred a greater sensitivity to the disruption of dopamine D2 receptors. Considering that a deficiency in a subpopulation of GABAergic neurons in the prefrontal cortex is also associated with schizophrenia (46), the loss of neocortical GABAergic neurons caused by the absence of PDK1-Akt signaling demonstrated in the present study might have important implications for the pathogenesis of schizophrenia.

Materials and Methods

Mouse Strains. Pdk1<sup>floxed</sup> Mash1<sup>1/2</sup> and nestin-CRE mice have been described (26, 43, 47, 48). All mice were maintained according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo. Details on other methods are available in SI Methods.

Statistical Analysis. Data are presented as means ± SEM, unless otherwise indicated. Values were compared with Student’s t test. A P value of <0.05 was considered statistically significant.

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Supporting Information

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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 fromCLEA 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), Akt3A (K179A/T308A/S473A), or AktKA homology domain (residues 4 to 129) and contains an added myristoylation site, Akt3A (K179A) were inserted into the BamHI 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-β-A-Luc (which contains 7 repeats of the E-box motif upstream of the β-actin gene promoter and the firefly luciferase gene) and pEFPBos-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′-TTGCCCACAGCCTTG-GCAGC-3′; Vglut1, 5′-TGGCTAAGGAGATTCTATG-3′ and 5′-CATTTGGAATGCGGGCTTTC-3′; Gad65, 5′-ACCAAGACATGCTTCTTATT-3′ and 5′-CATTTACACAGTTTGGACAACTTAC-3′; and Mash1, 5′-CTAGAACCTCTGAGCCTGCC-3′ and 5′-TCTTGCTTCCAAAGTCCATC-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.

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 Pdk1fl/flneo/flneo 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. 1C, 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. 3A) 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 mm² 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 mm² 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 (GSK3αβ KI, GSK3αβ (S21A);GSK3β (S9A)) were infected with the indicated retroviruses. The infected cells were cultured for 3 days in a differentiation condition. The percentage of βIII-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 mm² 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 mm² 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.)