Interaction of PDK1 with phosphoinositides is essential for neuronal differentiation, but dispensable for neuronal survival

Tinatin Zurashvili1, Lluis Cordón-Barris1, Gerard Ruiz-Babot1, Xiangyu Zhou1, Jose M Lizcano1, Nestor Gómez1, Lydia Giménez-Llort2 & Jose R Bayascas1#

1Institut de Neurociències & Departament de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, Barcelona E-08193 Spain
2Institut de Neurociències & Departament de Psiquiatria i de Medicina Legal, Universitat Autònoma de Barcelona, Barcelona E-08193 Spain

#Correspondence to JR Bayascas (joseramon.bayascas@uab.cat)
Phone: +34 93 5813762; Fax: +34 93 5811573

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Abstract.

The 3-phosphoinositide-dependent protein kinase-1 (PDK1) operates in cells in response to phosphoinositide 3-kinase activation and PtdIns(3,4,5)P3 production by activating a number of AGC kinases, including protein kinase B (PKB)/Akt. Both PDK1 and PKB contain pleckstrin homology domains that interact with the PtdIns(3,4,5)P3 second messenger. Disrupting the interaction of the PDK1 pleckstrin homology domain with phosphoinositides by expressing the PDK1 K465E knock-in mutation resulted in mice with reduced PKB activation. We explored the physiological consequences of this biochemical lesion in the central nervous system. The PDK1 knock-in mice displayed reduced brain size due to a reduction in neuronal cell size rather than cell number. Reduced BDNF-induced phosphorylation of PKB at Thr308, the PDK1 site, was observed in the mutant neurons, which was not rate-limiting for the phosphorylation of those PKB substrates governing neuronal survival and apoptosis, such as FOXO1 or GSK3. Accordingly, the integrity of the PDK1 PH domain was not essential to support the survival of different embryonic neuronal populations analyzed. In contrast, PKB-mediated phosphorylation of PRAS40 and TSC2, allowing optimal mTORC1 activation and BRSK protein synthesis, were markedly reduced in the mutant mice, leading to impaired neuronal growth and differentiation.
**Introduction**

During the development of the nervous system, among all the neuronal precursors initially produced during the neurogenesis stage, only those encountering the appropriate set of neurotrophic factors along with a complex coordinate of extracellular positional signals will be further selected to survive and differentiate (1). The PI3K/PKB axis is one of the critical intracellular signaling pathways that promotes neuronal survival by inhibiting the apoptotic cell death machinery in response to a number of extracellular stimuli (2). Thus, pharmacological inhibition of the PI3K catalytic activity causes neuronal cell death, whilst forced expression of constitutively active forms of the PKB/Akt kinase promotes the survival of many neuronal cell-types (3). PI3K also plays fundamental roles in regulating neuronal differentiation by defining the axon-dendrite axis through the activation of PKB (4). PKB promotes axon specification by inhibiting GSK3β (5). PKB also inhibits the TSC1-TSC2 complex, which antagonizes axon formation by inhibiting mTORC1 and restricting in this way the expression of the BRSK/SAD kinases (6), which are known to play fundamental roles in neuronal polarization in vivo (7,8).

However, mice lacking the neuronal Akt3/PKBγ isoform are viable and do not exhibit any overt phenotype, although display reduced brain size, with neurons more sensitive to apoptotic insults (9,10). Therefore, the contribution of kinases activated downstream of the PI3K cascade besides PKB cannot be overlooked. In this regard, a role for the closely-related kinases SGK (11) or RSK (12) in promoting neuronal survival, and for RSK in promoting neurite outgrowth (13) has been also proposed.

The 3-phosphoinositide-dependent protein kinase-1 (PDK1) elicits the cellular responses to growth factors, hormones and many other agonists that signal through phosphoinositide 3-kinase (PI3K) activation and phosphatidylinositol-3,4,5-
trisphosphate (PtdIns(3,4,5)P3) production by directly activating as much as twenty-three protein kinases of the AGC family. These include PKB/Akt, p70 ribosomal S6 kinase (S6K), serum- and glucocorticoid- induced kinase (SGK), p90 ribosomal S6 kinase (RSK) and protein kinase C (PKC) isoforms, which in turn regulate cell growth, proliferation, survival as well as metabolism (14,15). All these AGC kinases share structural homology and a common mechanism of activation based on the dual phosphorylation of two residues lying within two highly conserved motifs, namely the T-loop (Thr308 residue for PKBα) and the hydrophobic motif (Ser473 residue for PKBα). PDK1 acts as the master upstream kinase activating this set of AGC-kinases by phosphorylating their T-loop sites (16). The hydrophobic motif-kinase is different among the different AGC family members, although a prominent role for mTOR complexes has emerged (17). Thus, mTORC1 complex phosphorylates the hydrophobic motif of S6K isoforms (18,19) and novel PKC isoforms (20), whilst mTORC2 complex is the hydrophobic kinase for PKB (21), PKCα (22) and SGK isoforms (23).

PDK1 is expressed in cells as a constitutively active enzyme which is not modulated by any stimuli. Regulation of this intricate signaling network relies instead on the ability of PDK1 to specifically recognize and interact with its substrates (24). The interaction of PDK1 with most AGC-kinases needs the previous phosphorylation of their hydrophobic motifs, which become in this manner a substrate docking site for PDK1 binding (25). Activation of PKB/Akt isoforms represents an exception to this general mechanism. PKB isoforms are among all the PDK1-activated kinases the only ones possessing pleckstrin homology domains, a phosphoinositide binding domain that is also present in the PDK1 protein (26,27). The specific binding of the pleckstrin homology domain of PKB with PtdIns(3,4,5)P3 becomes rate-limiting for the translocation of PKB to the plasma membrane and co-localization with PDK1, where
PDK1 can then efficiently phosphorylate PKB at Thr308 (28,29) whilst mTORC2 phosphorylates the Ser473 site in the hydrophobic motif (21), resulting in maximal activation of the enzyme. The significance that the interaction of the PDK1 PH domain with phosphoinositides plays in the activation of PKB has been evaluated in vivo in the PDK1\(^{K465E/K465E}\) knock-in mice (30), which expresses a rationally-designed point mutant form of PDK1 that retains catalytic activity but is incapable of phosphoinositide binding (27). In tissues derived from these mice, PKB is still activated by growth factors, albeit to a reduced level (30-32), whereas the activation of the rest of PDK1 substrates proceeds normally. As a consequence, these mice are smaller, prone to diabetes (30) and protected from PTEN-induced tumourogenesis (32). The PDK1\(^{K465E/K465E}\) mouse is a genuine model in which PKB activation is only moderately reduced, which might originate from the ability of PDK1 to recognize the PKB Ser473 phospho-docking site in the absence of phosphoinositide binding (33). This genetic model has been proved instrumental in dissecting the PDK1 signaling (34) and has permitted to establish in T-cells that a PKB/Akt signaling threshold depending on PDK1-phosphoinositide interaction dictates specific cellular responses, such as cell migration, but not cell proliferation (31).

In the present study, we have employed the PDK1\(^{K465E/K465E}\) knock-in mice to explore the role that the interaction of the PDK1 PH domain with phosphoinositides plays in neuronal tissues. We found that the brain of the homozygous PDK1\(^{K465E/K465E}\) knock-in mice was reduced in size due to a reduction in cell size rather than cell number. In agreement with the maintenance of the number of cells, both the sensitivity of the mutant neurons to apoptosis induced by serum withdrawal, as well as the ability of different growth factors to support neuronal survival in the absence of serum, were preserved in the mutant neurons. The deficient activation of PKB and incomplete...
phosphorylation and inactivation of PRAS40 and TSC2 observed in the mutant neurons caused decreased mTORC1 activation, leading to reduced BRSK protein synthesis and deficient neuronal differentiation.
**Materials and Methods**

**Materials and constructs.** Protease-inhibitor cocktail, Thiazylol Blue Tetrazolium Bromide (MTT), Staurosporine, Dulbecco’s Modified Eagle’s Medium (DMEM), Basal Medium Eagle (BME) and Fetal Bovine Serum (FBS) were purchased from Sigma. Human recombinant BDNF was from Alomone and human recombinant IGF-1 was from Millipore. The Calbiochem Akti-1/2 (#124018), PI-103 (#528100), Rapamycin (#553210) and SB-216763 (#361566) inhibitors were purchased from Merk Millipore. Neurobasal, B27 Supplement, OptiMem and Lipofectamine™ 2000 were from Invitrogen. The HA-tagged human BRSK1 and BRSK2 (35) were sub-cloned into a pEIGW lentiviral vector that allows the expression of both GFP and BRSK1/2 from a bi-cistronic messenger.

**Antibodies.** The following antibodies were kindly provided by Prof. Dario Alessi from the University of Dundee. All were raised in sheep and affinity purified on the appropriate antigen. The PKBα total antibody was raised against the sequence RPHFPQFSYSASGTA corresponding to residues 466–480 of rat PKBα; The total TSC2 antibody was raised against a sequence encompassing residues 1719-1814 of mouse TSC2; the total PRAS40 antibody was raised against the peptide DLPRPRLNTSDFQKLKRKY corresponding to residues 238-256 of human PRAS40; the total NDRG1 antibody was raised against the recombinant human NDRG1 protein expressed in *E. coli*. Affinity-purified polyclonal BRSK1 or BRSK2 specific antibodies were raised in sheep against the peptide SPRRGPPDKKLLATNGTPLP corresponding to the C-terminal residues 774–794 of human BRSK1 and the peptide LSWGAGLKGQKVATSYESSL encompassing residues 655–674 of human BRSK2, as described (36). The phospho-PKB Thr308 (#9275), phospho-PKB Ser473 (#9271), phospho-S6K Thr389 (#9205), total S6K (#9202), phospho-S6 ribosomal protein
Ser235/236 (#2211), total S6 ribosomal protein (#2217), phospho-p44/42 MAPK
Thr202/Tyr204 (#9101) total p44/42 MAPK (#9102), phospho-GSK3α/β Ser21/9
(#9331), phospho-FOXO1 Ser256 (#9461), phospho-FOXO1 Thr24 (#9464), total
FOXO1 (#2880), phospho-RSK Ser380 (#9335), phospho-RSK Thr573 (#9346), total
RSK1/2/3 (#9355), phospho-TSC2 Thr1462P (#3611), phospho-PRAS40 Thr246
(#2997), phospho-NDRG1 Thr346 (#5482), phospho-TrkB Tyr706/707 (#4621) and
total TrkB (#4603) were purchased from Cell Signaling Technology. The pan-PDK1
site antibody from Cell Signaling Technology (#9379) recognizes the phosphorylated
Thr229 of S6K in cell extracts (37). The total GSK3α/β (sc-7291) and the phospho-RSK
Thr227 (sc-12445) were purchased from Santa Cruz Biotechnology. Appropriate
secondary antibodies coupled to horseradish peroxidase were from Pierce. For the
immunofluorescence analysis, the Anti-Tau-1 monoclonal antibody was purchased from
Millipore (#MAB3420), the Pan-Axonal Anti-Neurofilament H monoclonal antibody
from Covance (#SMI-312R), and the rabbit Anti-MAP2 polyclonal antibody (#M3696)
from Sigma; Alexa Fluor 594-conjugated goat anti-rabbit (#A11072) and Alexa Fluor
488-conjugated goat anti-mouse (#A11017) fluorescent secondary antibodies were from
Invitrogen.

Mice. The generation and genotyping of the PDK1\text{K465E/K465E} knock-in mice expressing
the single-aminoacid substitution of lysine 465 to glutamic acid in the PDK1 PH
domain have been described previously (30). Mice were maintained in the Animal
House Facility of the Universitat de Lleida under standard husbandry conditions. All
animal studies and breeding were approved by the Universitat Autònoma de Barcelona
ethical committee and performed under a Generalitat de Catalunya project license.

Primary cultures. Cerebral cortical or hippocampal tissues were dissected from
PDK1\text{+/+} and PDK1\text{K465E/K465E} littermate mice at embryonic day 15.5, and the cells were
enzymatically dissociated in Krebs Ringer Buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 25 mM NaHCO3, 14.3 mM glucose) containing 0.25 mg/ml of trypsin for 10 min at 37°C, and then were mechanically dissociated in Krebs Ringer Buffer containing 0.08 mg/ml DNase and 0.52 mg/ml trypsin inhibitor by gentle pipetting using a fire-polished Pasteur pipette to produce a single cell suspension. Cells were then centrifuged and resuspended, counted with the Scepter™ 2.0 Handheld Automated Cell Counter (Millipore) and finally diluted in DMEM complemented with 2 mM L-Glutamine, 0.25 mg/ml penicillin/streptomycin, 10% FBS. The cortical cells were then plated onto poly-D-Lysine (50 μg/ml) coated 24-well plates for cell viability studies, or 6-well plates for western blot analysis, at a density of 15 X 10⁴ cells/ml, whereas the hippocampal cells were plated onto poly-D-Lysine (150 μg/ml) coated 12-mm diameter glass cover-slips at a density of 5 X 10⁴ cells/ml. The cells were allowed to attach to the plate for two hours and then the medium replaced by Neurobasal complemented with 2 mM L-Glutamine, 0.25 mg/ml of penicillin/streptomycin, 2% B27 Supplement. Cells were maintained at 37°C in a humidified incubator containing 5% CO2 in normoxia conditions.

Cerebellar granule cell cultures were prepared from dissociated cerebella of 8 day old mice by mechanically chopping the cerebellum followed by trypsin digestion and trituration as described in the previous paragraph. Cells were plated in BME supplemented with 25 mM KCl, 10% FBS, 0.25 mg/ml of penicillin/streptomycin. 10 μM Cytosine-β-D-arabinofuranoside was added to the culture after 24 h of seeding to prevent the proliferation of non-neuronal cells. Cells were plated onto 48-well culture plates coated with 10 μg/ml Poly-L-Lysine at a density of 1.35 X 10⁶ cells/ml.

**Trophic deprivation and drug treatment.** Survival experiments were performed at DIV 6. Cells were washed twice with serum-free DMEM and then incubated for 24 h in...
serum-free Neurobasal supplemented with 2 mM L-Glutamine, 0.25 mg/ml of penicillin/streptomycin. The experimental controls included sham treatments consisting of two washes with serum-free media and incubation with the same conditioned media. BDNF and IGF-1 were diluted in DMEM without any supplement. The inhibitors Akti-1/2, PI-103, SB-216763, staurosporine and rapamycin were dissolved in dimethyl sulfoxide (DMSO). For viability and apoptosis analysis, growth factors and inhibitors were added at the onset of trophic deprivation. For western blot analysis, cells were pre-treated for 30 min with inhibitors and then stimulated with BDNF as indicated. Evaluation of cell viability. Cell viability was determined by the MTT reduction assay. Briefly, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) salt was added to the cell culture at a final concentration of 0.5 mg/ml. Plates were then returned to the incubator for 45 min. After incubation, the medium was aspirated and the resulting formazan crystals were dissolved by mixing with 300 μl of DMSO. Absorbance intensity was measured at 570 nm, with background measured at 690 nm, using a spectrophotometer running the Labsystem Multiskan® software. Quantification of apoptosis. Cells were fixed in 2% paraformaldehyde, stained with 1 μg/ml of the DNA dye Hoechst 33342, and then visualized under the fluorescence microscope. Apoptosis was quantified at each condition point by scoring the percentage of apoptotic cells in the adherent cell population. Cells exhibiting fragmented or condensed nuclei were scored as apoptotic whilst cells showing uniformly stained nuclei were scored as viable. At least 300 cells from 6 randomly selected fields per well were counted. Transfection. 2.5 x 10⁴ embryonic hippocampal neurons grown for two days in 12-mm diameter 24-well plates were transfected with 0.7 μl of the Lipofectamine™ 2000 reagent diluted in 50 μl of OptiMem plus 1 μg of the indicated DNA diluted again in 50
μl of Optimem. After 3 h, the transfection media was replaced by conditioned media, and the axon length of the GFP-expressing cells evaluated at day in vitro 4 as described in the corresponding section.

**Generation of protein extracts and western blot analysis.** Primary cortical neurons were cultured for six days, then incubated for 4 h in Neurobasal without B27, and subsequently stimulated with the indicated agonists as described in the figure legends. The neurons were lysed at the indicated time points in ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1mM EDTA, 1mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 0.27 M sucrose, 1% (w/v) Triton-X 100, 0.1% (v/v) 2-mercaptoethanol, 1:100 Protease Inhibitor Cocktail) and centrifuged at 4°C for 10 min at 13,000 X g. Tissue extracts were prepared by homogenizing the frozen tissue on ice in a 10-fold volume excess of ice-cold lysis buffer using the Polytron and then centrifuged at 4°C for 10 min at 13,000 X g to remove insoluble material. The supernatants were aliquoted, frozen in liquid nitrogen and stored at -20°C until use. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard. The activation state of the different pathways analyzed was assessed by immunoblotting extracts (10 μg) with the indicated antibodies and detected with the appropriate horseradish peroxidase-conjugated secondary antibodies. Membranes were incubated with the enhanced chemiluminescence reagent (ECL) and either exposed to Super RX Fujifilm and developed, or detected using a GeneGnome HR detection system (Syngene; Cambridge, UK) and quantified using the Image J software.

**Determination of organ volume and cell size.** Organ volume was determined using the Cavalieri method (38) on brain paraffin sections of 8 μm collected at systematically spaced locations (k=96 μm) from a random starting position. The sections were
photographed with a Nikon SMZ800 stereomicroscope at 2 X magnification using a
digital camera. A square lattice grid was then overlaid on the picture using the program
Photoshop version vCS5.1 and the number of intersections (P) hitting the organ was
scored. The organ volume was then estimated using the equation \( \sum P \times d^2 \times k \), in which
d^2, the distance between each point of the square lattice grid squared was 0.14792 mm^2.
The number and size of the cells was determined on E15.5 dissociated cortex and
hippocampal tissues with the Scepter™ 2.0 Handheld Automated Cell Counter

**Immunocytochemistry.** Dissociated hippocampal cells were cultured on cover slips for
the indicated experimental days in vitro and then fixed with 4% paraformaldehyde in
PBS for 20 min at room temperature. Fixative solution was rinsed 3 times with PBS for
5 min, and then the cells were permeabilized with 0.02% saponin in PBS for 7 min at
room temperature and blocked in 0.01% saponin, 5% BSA, 10 mM Glycine in PBS for
1 h. Primary antibodies were diluted 1:200 in PBS supplemented with 0.01% saponin,
1% normal goat serum and incubated overnight at 4°C. Cells were then washed three
times with PBS for 10 minutes. Appropriate secondary antibodies conjugated to
Alexa594 or Alexa488 fluorescent dyes were used at a concentration of 1:400, and
nuclei were stained with 1 μg/ml Hoechst 33342. Cover slips were then mounted with
FluorSave Reagent on microscope slides for further analysis.

**Immunohistochemistry.** Three PDK1^{+/+} and three PDK1^{K465E/K465E} matched littermate
mice were anesthetized by intra-peritoneal injection with a ketamine/xylocaine mixture
and then intracardially perfused with 0.9% NaCl followed by 4% buffered
paraformaldehyde. Brains were extracted and post-fixed with the same solution for 2 h,
washed in phosphate buffer 0.1 M pH 6.0 for 2 h and preserved in a 70% ethanol
solution at 4°C. All the six specimens were then embedded in the same paraffin block
and sliced into 5 μm thick coronal sections with a Leica RM2255 microtome. Paraffin-embedded sections were incubated for 2 h at 60°C and then rehydrated through a series of 2 washes with xylene for 5 min, 2 washes with 100% ethanol for 3 min, 1 wash with 96% ethanol for 3 min, 1 wash with 70% ethanol for 3 min, 1 wash with 50% ethanol for 5 min and 1 wash with water for 1 min. Sections were then boiled 10 min in 10 mM sodium citrate pH 6 for antigen retrieval and cooled down for 30 min on ice. Samples were blocked in Tris Buffer Saline (TBS, 50 mM Tris pH 7.5, 150 mM NaCl) containing 0.02% Triton and 5% goat serum for 30 min, and incubated overnight at 4°C with primary antibodies diluted in the same blocking solution (1:300 for the rabbit anti-MAP2 antibody and 1:2000 for the mouse monoclonal anti-pan axonal marker SMI-312R). Sections were rinsed with TBS buffer and detected with the Alexa488-conjugated anti-mouse (1:400) and Alexa594-conjugated anti-rabbit (1:300) secondary antibodies for 1.5 h at room temperature. Tissue autofluorescence was removed by incubation with Sudan Black for 10 minutes (0.3% w/v Sudan Black in 70% ethanol). Sections were counterstained with Hoechst and mounted with FluorSave Reagent. Immunostained sections were photographed with a Nikon Eclipse 90i epifluorescent microscope and the captured images analyzed and processed with the ImageJ 1.42q (Wayne Rasband, National Institutes of Health, USA) and the Fiji (http://pacific.mpi-cbg.de/wiki/index.php/Main_Page) softwares.

**Evaluation of differentiation.** For differentiation analysis of the cortical neurons, cells were seeded at a reduced density of 75000 cells/ml. Under this condition, most neurons did not contact with neighboring cells, allowing the measurement of neurite development of individual neurons. Images were acquired at 20 X magnification on random fields by using an inverted microscope equipped with a high sensibility camera Hamamatsu Orca-Er at the maximal resolution of the microscope. The total neurite
length per cell was measured by tracing all the neurites on each individual neuron from the cell body to the tip with the Adobe Photoshop vCS5.1 software, and the number of pixels converted to micrometers. The cell diameter was defined using the same technique by measuring the major axis of the neuronal cell body. The number of neurites per cell and number of branching points per neurite were also determined by direct counting on the acquired images.

For differentiation analysis of the hippocampal cells, images were obtained with an epifluorescent microscope (Nikon Eclipse 90i) interfaced to a DXM 1200F camera at 20 X magnification. Images for the red and green channels were taken simultaneously. Neuronal dendrites were identified by immunostaining with the dendritic marker MAP2, whereas the axon was defined as a neurite whose length is 2 times longer than the other neurites and also is immunoreactive for the axonal marker Tau-1. To measure the axonal elongation, each particular axon was manually traced followed by automatic length calculation with the MetaMorph® image analysis software v6.1.

Statistical analysis. Statistical significance was determined using the Student's t test analysis. \(*p < 0.05, **p < 0.005\) between categories or conditions as indicated in the figure legends.
Results

Reduced brain size of PDK1\(^{K465E/K465E}\) mice.

Mice homozygous for the PDK1 K465E knock-in alleles were previously reported to be viable, fertile and healthy, although displayed a 35% reduction in body weight compared to wild type mice (30). We observed the homozygous PDK1\(^{K465E/K465E}\) genotype at reduced Mendelian distribution from heterozygous crosses, both at embryonic day 15 and at birth (19.8 % and 16.0 % respectively), thereby indicating that the PDK1 K465E mutation resulted in partial embryonic lethality (Fig. 1A). The growth deficiency of the PDK1\(^{K465E/K465E}\) mice might initiate early during development, as we observed that at E15.5, the PDK1 knock-in embryos were already 20% smaller than their control littermates (Fig. 1B), with brain size also reduced to scale (Fig. 1C). The small phenotype was further exacerbated after the second week of age, as the body weight of the PDK1\(^{K465E/K465E}\) mice was 30-35% reduced when compared to the PDK1\(^{+/+}\) mice from three weeks of age (Fig. 1B) and during their adulthood (data not shown). To establish whether the reduction in the embryonic brain volume could be attributed to a reduction in cell size or number, the volume of the neuronal soma and the number of neuronal cells purified at E15.5 from both the embryonic cortex and the hippocampus were determined. Whilst the number of cortical and hippocampal neurons was not significantly different between genotypes, the soma was 20% reduced in volume in the cortical (Fig. 1D) and hippocampal (Fig. 1E) mutant cells when compared to controls, thereby demonstrating that the small size of the PDK1\(^{K465E/K465E}\) mice brain is mostly due to a reduction in cell size rather than cell number.

Binding of PDK1 to PtdIns(3,4,5)P\(_3\) is not essential to support neuronal survival.

We next compared the protective effects that BDNF elicited on the survival of PDK1\(^{+/+}\) and PDK1\(^{K465E/K465E}\) cortical neurons deprived from growth factors. In the control
cultures, trophic factor deprivation compromised the cell viability, as denoted by a 50% decrease in the MTT reduction values, which was accompanied by a three-fold increase in the number of apoptotic cells when compared to the untreated cultures. BDNF stimulation markedly recovered the cell viability and decreased the number of apoptotic-dying cells of the serum-deprived cortical cultures (Fig. 2A). Unexpectedly, trophic factor withdrawal compromised the neuronal viability to the same extent in the PDK1+/+ and the PDK1K465E/K465E cultures, which was equally rescued by BDNF treatment in the two genotypes, thereby suggesting that the interaction of PDK1 with phosphoinositides is not essential for the neuroprotective actions of BDNF, at least in cortical neurons. We extended our observations to other neuronal populations, and found that IGF-1 prompted the survival of cerebellar granule cells deprived from serum and potassium to the same level in the PDK1+/+ and the PDK1K465E/K465E cultures (Fig. 2B). Moreover, the PDK1+/+ and the PDK1K465E/K465E cortical neurons exhibited the same sensitivity to apoptotic stimuli such as staurosporine (Fig. 2C). We also cultured the cortical neurons in the presence of suboptimal doses of either BDNF or IGF-1, and found that the two trophic factors elicited a dose-dependent neuroprotective action that was similar in both control and mutant cultures (Fig. 2D). Because the interaction of PDK1 with phosphoinositides is important for PKB activation (30), PKB/Akt is a major regulator of neuronal survival (2), but the neuronal survival responses were still not affected in the PDK1K465E/K465E mice (Fig. 2), we assessed whether inhibition of PKB compromised the protective role of BDNF against serum deprivation. To that end, we employed the Akti-1/2 inhibitor, which specifically targets PKBα and PKBβ. The treatment of cortical cultures with the Akti-1/2 compound at specific doses that markedly prevented PKB phosphorylation at both Thr308 and Ser473 sites, did not affect the survival responses elicited by BDNF. As a control, the cortical cultures were
also treated with the PI3K specific inhibitor PI-103, and found that the inhibition of 
PI3K totally abolished both the recovery of the cell viability and the inhibition of  
apoptosis induced by BDNF (Fig. 3).  

**Mutation of the PDK1 PH-domain impairs BDNF-mediated PKB activation.**

To define the importance of the PDK1-PtdIns(3,4,5)P$_3$ interaction on the ability of 
PDK1 to activate PKB, primary cultures of cortical neurons derived from littermate  
PDK1$^{+/+}$ and PDK1$^{K465E/K465E}$ embryos were stimulated with BDNF for the indicated 
time points (Fig. 4A). As a control of the stimulation, the activation of the BDNF 
receptor TrkB was monitored by measuring its phosphorylation at the activation loop 
residues Tyr706/707, which was very rapid and sustained in both control and mutant 
cell extracts. In the PDK1$^{+/+}$ cells, BDNF induced a clear activation of PKB, as judged  
by the level of phosphorylation of the two activating residues, Thr308 and Ser473,  
which reached the maximum after 5 minutes and was then sustained for up to 30  
minutes. By contrast, the phosphorylation of PKB at Thr308, the PDK1 site, was  
significantly reduced in the PDK1$^{K465E/K465E}$ mutant neurons during the first 15 min of  
stimulation and was then detected at nearly normal levels after 30 min, whereas the  
phosphorylation of PKB at Ser473, the mTORC2 site, was not affected in the mutant  
cells (Fig. 4A and C). Consistent with a reduction in the ability of the PDK1 K465E  
mutant protein to activate PKB, the phosphorylation levels of some PKB substrates at  
their specific PKB sites, namely PRAS40 at Thr246 and TSC2 at Thr1462, were also  
significantly reduced in the mutant extracts. By contrast, the PKB-specific  
phosphorylation of GSK3$\alpha/\beta$ at Ser21/9 and FOXO1 at Thr24 and Ser256 were not  
affected by the PDK1 mutation (Fig. 4A). The moderate changes that the PDK1 PH  
domain mutation impinges on the PKB downstream signaling pathways were time- and  
dose-dependent, and were better observed at low concentrations of BDNF (Fig. 4B).
Reduced activation of S6K in the PDK1<sup>K465E/K465E</sup> cortical neurons.

Activation of S6K involves the phosphorylation of the Thr389 residue within the S6K hydrophobic motif by mTORC1, followed by the phosphorylation of the Thr229 residue in the S6K activation loop by PDK1. Therefore, mTORC1 regulation greatly dictates S6K activation. PKB itself contributes to the activation of mTORC1 by phosphorylating and inhibiting two mTORC1 inhibitory proteins, namely PRAS40 and TSC2. Accordingly, although S6K is a docking site-dependent PDK1 substrate, the reduced phosphorylation of PKB, PRAS40 and TSC2 proteins observed in the BDNF-stimulated PDK1<sup>K465E/K465E</sup> cortical neurons (Fig. 4) resulted in deficient activation of mTORC1, as judged by the reduced phosphorylation of S6K at Thr389, albeit this defect was very transient and only detectable at 5 minutes of BDNF treatment. As a consequence, PDK1 phosphorylation of the Thr229 residue was also reduced, leading to decreased S6K activation, as revealed by the impaired phosphorylation of the ribosomal S6 protein at Ser235 (Fig. 5A).

Normal activation of RSK in the PDK1<sup>K465E/K465E</sup> neurons.

RSK is activated downstream of the ERK signaling pathway in a multi-step phosphorylation sequence. ERK activates the C-terminal kinase domain (CTKD) of RSK by phosphorylating the T-loop Thr573 residue. The activated CTKD auto-phosphorylates the RSK hydrophobic motif at Ser380, thereby creating the docking site for PDK1, which can then phosphorylate the T-loop residue Ser227 in the N-terminal kinase domain (NTKD), leading to its activation (39). BDNF induced in cortical cells a rapid phosphorylation of ERK1/2 at Thr202/Tyr204, which reached the maximum at 5 minutes and was sustained for 30 minutes. As expected, this was not affected by the PDK1 K465E mutation. That was accompanied by a robust induction of RSK
phosphorylation at Thr573, Ser380 and Ser227, which was similar in both control and mutant cells (Fig. 5B).

**Reduced phosphorylation of the SGK1 substrate NDRG1.**

Activation of SGK1 encompasses the phosphorylation of the hydrophobic motif at Ser422 by mTORC2, which primes the phosphorylation of the T-loop at Thr256 by PDK1. The n-myc downstream-regulated gene (NDRG) family members were identified as the first specific physiological substrates of the SGK isoforms (40), and the phosphorylation of the NDRG1 protein at Thr346/356/366 sites, lying in a C-terminal decapeptide repeated sequence, is used as a read out of the SGK1 activity (23). The NDRG proteins play important roles in the development of the central nervous system. Mutations in the NDRG1 gene causes motor and sensory neuropathy in humans (41), whereas NDRG1 deficient mice exhibited peripheral nerve degeneration (42). NDRG2 levels are upregulated in Alzheimer Disease (43), and NDRG4 deficient mice exhibit spatial learning deficits and vulnerabilities to cerebral ischemia (44). However, the physiological relevance that NDRG phosphorylation by SGK1 plays in neurons is largely undefined. We used antibodies recognizing the phosphorylated C-tail repeats of the NDRG1 protein, and found that BDNF rapidly induced the phosphorylation of NDRG1 at the SGK1 sites in PDK1+/+ cortical neurons, which was significantly reduced in the PDK1K465E/K465E cells at 5 minutes of BDNF treatment (Fig. 6A). This observation was puzzling because a role for the PDK1-phosphoinositide interaction in activating SGK1 could not be envisaged. Another scenario would be that the PKB isoforms contributed to the phosphorylation of NDRG1 at Thr346/356/366, at least in neurons. We tested this notion by stimulating cortical neurons with BDNF in the presence of the Akti-1/2 inhibitor, and found that BDNF-induced phosphorylation of NDRG1 at Thr346/356/366 was greatly reduced by the Akti-1/2 compound at doses that did not
affected other closely related kinases such as RSK (Fig. 6B). Since SGK1 phosphorylation primes NDRG1 for GSK3 phosphorylation at positions n-4 (40), the phosphorylation of NDRG1 at Ser Thr342/352/362 by GSK3, which could have been enhanced by the PDK1 K465E mutation, might have interfered with the recognition of NDRG1 by the phospho-Thr364/356/366 antibody. Treatment of BDNF-stimulated cells with two independent GSK3 inhibitors, namely lithium and SB-216763, caused an increase in the electrophoretic mobility of the NDRG1 protein, which was compatible with a decrease in the abundance of the hyperphosphorylated NDRG1 species. By contrast, inhibitors of GSK3 did not significantly affect the intensity of the phospho-Thr346/356/366 signal (Fig. 6B), thereby indicating that the phosphorylation of the GSK3 sites were not masking the NDRG1 phospho-Thr346/356/366 antibody epitope, and further suggesting a direct role of PKB in phosphorylating neuronal NDRG1 at the SGK1 sites.

PtdIns(3,4,5)P3 binding to PDK1 promotes neuronal differentiation through the PKB/mTORC1/BRSK pathway.

We showed that disrupting the interaction of PDK1 with PtdIns(3,4,5)P3 in the PDK1K465E/K465E mice affected the activation of PKB by BDNF, leading to the inhibition of the downstream mTORC1 and S6K signaling pathways. Since mTORC1 plays fundamental roles in regulating neuronal morphogenesis (45), we aimed to determine whether the PDK1K465E/K465E mice exhibited alterations in neuronal morphology. To that end, primary cortical neurons derived from PDK1+/+ and PDK1K465E/K465E E15.5 embryos were allowed to differentiate in culture, and the complexity of the neuronal processes measured at different days in vitro (DIV) by scoring the length of the neurites, the number of neurites and the number of branching points. The ability of the PDK1K465E/K465E embryonic cortical neurons to differentiate in culture was significantly
reduced at DIV3, which was further aggravated at DIV4 (Fig. 7A). Indeed, the neurites from the PDK1<sup>K465E/K465E</sup> embryonic cortical neurons were at DIV3 20% shorter when compared to control PDK1<sup>+/+</sup> cells; the rate of neurite outgrowth was then nearly null in the PDK1<sup>K465E/K465E</sup> cultures from DIV3 to DIV4, resulting in neurites that were as much as 40% shorter in the PDK1<sup>K465E/K465E</sup> cultures by DIV4 (Fig. 7B). To confirm than the reduced length of the PDK1<sup>K465E/K465E</sup> cortical neurons could not be the consequence of the reduced size of the mutant cells, we also measured the major axis of the neuronal soma, and found than this was only 10% shorter in the mutant neurons (Fig. 7C). We also found the number of neurites and branching points of the mutant neurons similar to that of the controls, whereas the percentage of undifferentiated cells was significantly higher in the mutant cultures when compared to the controls (data not shown).

We also took advantage of the widely used model of dissociated hippocampal neurons in culture for studying neuronal polarization (46). We determined axonogenesis during the differentiation of hippocampal primary cultures, as detected by immunocytochemistry with the Tau-1 specific axonal marker and the MAP2 dendritic marker. Axon formation and growth was markedly impaired in the PDK1<sup>K465E/K465E</sup> cells (Fig. 8A). While most of the PDK1<sup>+/+</sup> hippocampal neurons exhibited a differentiated axon by DIV 3, the PDK1<sup>K465E/K465E</sup> hippocampal neurons did not reach this stage of differentiation until DIV 4, and still the percentage of cells exhibiting one axon was significantly lower in the mutant cultures when compared to the control ones, both at DIV3 and DIV4 (Fig. 8B). Moreover, the length of the axons was consistently reduced by 25% in the PDK1<sup>K465E/K465E</sup> hippocampal neurons when compared to the PDK1<sup>+/+</sup> controls at all the time points analyzed (Fig. 8C), whilst the cellular soma was only 5% shorter in the mutant hippocampal cells (data not shown). These results reveal
that the interaction of PDK1 with phosphoinositides is required for both cortical and hippocampal neuronal morphogenesis.

To assess whether modulating PKB activity directly affected neuronal differentiation, primary cultures of hippocampal neurons were allowed to differentiate in vitro for four days in the presence or absence of the Akti-1/2 isoform specific inhibitor, and the axon specification and length measured at different time points. Axon formation and growth was drastically impaired by the Akti-1/2 compound, with the percentage of cells exhibiting one differentiated axon significantly reduced in the mutant cultures compared to the control ones (Fig. 8D). Moreover, the length of the axons was also reduced in the Akti-1/2-treated hippocampal neurons to a similar extent than that observed in the PDK1K465E/K465E mutant cells (Compare Fig. 8E with 8C).

In order to assess whether the PKB-dependent deficient mTORC1 activation was mainly responsible for the described phenotypes, we also treated hippocampal primary cultures with the mTORC1 specific inhibitor rapamycin, and found that complete inhibition of the mTORC1 pathway compromised neuronal polarization to a similar extent to that observed in the Akti-1/2 treated cells (Fig. 8D). By contrast, axonal elongation was even more severely impaired by rapamycin than by the Akti-1/2 inhibitor, resulting in axons that were as much as 50% shorter than those of the untreated cells (Fig. 8E).

The tuberous sclerosis complex proteins TSC1 and TSC2 have been shown to control axon formation by modulating mTORC1 activity and the synthesis of specific proteins that are required for neuronal polarization, such as the brain-specific kinase (BRSK)-1 and -2 (6). The BRSK1 and BRSK2 kinases are expressed during the differentiation of the cortex and the hippocampus, where they play fundamental roles in establishing proper neuronal polarity (7,8). In agreement with that notion, we observed
that both the BRSK1 and BRSK2 proteins were expressed at low levels in primary cultures of cortical neurons from the first day in vitro, and that the levels of expression were then induced by day in vitro three. Strikingly, expression of the BRSK proteins was significantly reduced in the PDK1K465E/K465E cortical neurons from the first day in culture, which progressively recovered to nearly normal levels along the in vitro differentiation period (Fig. 9A). In agreement with this observation, BRSK1 and BRSK2 protein levels were also markedly reduced in brain extracts from the PDK1K465E/K465E E15.5 embryos, whilst BRSK1 and BRSK2 protein expression was found to be similar in the adult brain of both the PDK1K465E/K465E and the PDK1+/+ mice. By contrast, phosphorylation of PKB at Thr308, PRAS40 at Thr246 and TSC2 at Thr1462 was consistently reduced in the mutant protein extracts during development and in the adult (Fig 9B). BRSK1 expression was also reduced in PDK1K465E/K465E hippocampal neurons when compared to PDK1+/+ control cells, and the treatment of these neurons with rapamycin clearly decreased the levels of BRSK1 protein (Fig. 9C). Altogether, these data suggest that the axonal phenotype observed in the PDK1K465E/K465E neurons is likely due to deficient PKB-mediated mTORC1 activation and blunted stimulation of BRSK expression. We tested this notion by re-expressing BRSK1 and BRSK2 in PDK1K465E/K465E hippocampal neurons, and found that this rescued the growth deficiencies of the knock-in neurons, whereas it had little effect on the axonal elongation of wild type cells (Fig. 9D), thereby providing strong evidence on the role of the BRSK kinases are key downstream targets mediating the actions of PDK1-phosphoinositide interaction in axon morphogenesis.
Discussion.

In the PDK1\textsuperscript{K465E/K465E} mice neurons, activation of PKB was partially inhibited due to incomplete phosphorylation of PKB at the PDK1 site. Phosphorylation of the PKB Thr308 site was previously shown to be reduced by three- to four-fold in the insulin-responsive tissues of the PDK1\textsuperscript{K465E/K465E} mice (30). We found the impact that the PDK1 K465E mutation has on PKB Thr308 phosphorylation less severe in neuronal tissues than in insulin-responsive tissues, since BDNF–induced phosphorylation of PKB at Thr308 was only reduced by two-fold in the PDK1\textsuperscript{K465E/K465E} cortical neurons (Fig. 4C). When the PDK1 K465E knock-in mutation was first characterized, the observation that PKB was still phosphorylated at Thr308 in the absence of PDK1-PtdIns(3,4,5)P\textsubscript{3} interaction was unexpected. More recently, it has been proposed that the phosphorylation of PKB at Thr308 detected in the PDK1\textsuperscript{K465E/K465E} knock-in ES cells relies on the PIF-pocket mechanism (33). It would be interesting to study whether this PIF pocket-dependent activation of PKB operates more efficiently in neurons than in other cell types. In this regard, the activation of the PIF pocket-dependent kinases S6K, RSK or SGK, which occurs more slowly than the activation of PKB in several cell types, parallels in neurons the activation of PKB, and reaches the maximum at five minutes of BDNF treatment.

We show that the reduced levels of PKB activation are sufficient to support the phosphorylation and inhibition of some cellular targets promoting apoptosis, such as the FOXO1 transcription factor (47) or the GSK3 kinase (48). By contrast, the reduced levels of PKB activation achieved in the PDK1\textsuperscript{K465E/K465E} neurons are rate limiting for PRAS40 and TSC2 phosphorylation. The inefficient phosphorylation of PRAS40 and TSC2 resulted in reduced activation of mTORC1, leading to impaired phosphorylation of the S6K at Thr389 within the hydrophobic motif. This most likely compromised the

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interaction of PDK1 with S6K, resulting in reduced S6K phosphorylation at Thr229 in the activation loop and diminished S6 protein phosphorylation, thereby explaining why the activation of a docking site-dependent kinase appears to be affected by the PDK1-phosphoinositide binding. Moreover, we could bypass this requirement by stimulating mTORC1 with aminoacids, which rendered normal levels of S6K and S6 protein phosphorylation in the mutant cells (data not shown).

The SGK isoforms are phosphorylated by PDK1 following the phosphorylation of their hydrophobic motif by mTORC2 (23). Because the agonist-stimulated mTORC2 activation, as inferred from the levels of PKB Ser473 phosphorylation detected in the PDK1K465E/K465E neurons, is not affected by the PDK1 mutation, and PDK1 can phosphorylate and activate SGK in a PtdIns(3,4,5)P3 independent-manner (49), SGK activation was not expected to be affected in the mutant cells. In this regard, insulin-induced phosphorylation of NDRG1 at the SGK1 sites was previously shown to be preserved in the muscle and the liver of the PDK1K465E/K465E mice (30). By contrast, phosphorylation of NDRG1 at Thr346/356/366 was reported to be severely impaired in several tissues of the SGK1 knockout mice, including spleen lung, liver and muscle (40). However, we found reduced BDNF-induced phosphorylation of NDRG1 at Thr346/356/366 in the PDK1K465E/K465E neurons. Although we failed to detect the phosphorylation of SGK1 at the Thr256 and Ser422 sites, it is unlikely that the phosphorylation of this activation sites by PDK1 or mTORC2 requires the binding of PDK1 to phosphoinositides. By contrast, we provided evidences suggesting that NDRG1 can be phosphorylated by PKB, at least in BDNF-stimulated cortical neurons. Moreover, we observed reduced phosphorylation of NDRG1 in PDK1K465E/K465E brain extracts (Fig. 6C). In fact, PKB was originally shown to be capable of marginally phosphorylating the NDRG2 isoform at the Thr348 residue (40). In this regard, two of
the residues in the NDRG1 sequence that conform to the specific SGK1 phosphorylation signature, namely Ser at position n+1 and Glu at n+2, which were probed to be deleterious to phosphorylation by PKB (50), are not conserved in the NDRG2 sequence. By contrast, the PRAS40 Thr246, which is a bona fide PKB phosphorylation site, possesses Ser at position n+1 and Asp at n+2 (Fig. 6D).

Activation of RSK by PDK1 also relies on a phospho-docking site interaction in which the phosphorylation of the RSK hydrophobic motif is under the control of the ERK pathway. BDNF triggers in neurons the activation of both the PI3K and the ERK signaling pathways. As expected, the activation of RSK isoforms by BDNF, as monitored by the phosphorylation of the ERK site, the hydrophobic motif autophosphorylation site, and the T-loop PDK1 site, proceeded normally in the PDK1K465E/K465E neurons.

The PDK1K465E/K465E mice are smaller, with reduced cell volume resulting from deficient activation of the PKB/mTORC1/S6K signaling axis (30). The small size phenotype is common in mice lacking different elements of this signaling network, including the PKBα deficient mice (51,52), the mTOR hypomorphic mice (53), the S6K1 deficient mice (54), or the S6K1 and S6K2 double knockout mice (55). In the PDK1K465E/K465E mice, the volume of the brain was 20% reduced when compared to PDK1+/+ controls. Mice lacking the PKBγ gene, which is mainly expressed in neuronal cells, displayed a selective 20-25% reduction of the brain size, whereas the rest of organs analyzed were of normal size (9,10). In the PKBα knockout mice, a general reduction in the body size that was accompanied by a proportional reduction in the size of different organs including the brain was also demonstrated (9). Whilst the reduced brain size was mainly attributed to a decrease in the number of cells in the PKBα knockout mice, the reduced brain volume observed in the PKBγ knockout mice was
proposed to be mainly due to the presence of smaller cells (9). Likewise, our data
clearly demonstrates that the reduced brain size of the PDK1<sup>K465E/K465E</sup> mice is due to a
reduction of the cell size, whereas the number of neurons is unaffected. It would be
interesting to define whether the number and size of the neurons are specifically
controlled by different PKB isoforms, or whether the differences observed among the
different transgenic mice could be explained in the terms of whole PKB activation
levels.

One salient finding derived from these studies is that in PDK1<sup>K465E/K465E</sup> knock-in mice, the survival responses of cortical, hippocampal and cerebellar neurons to
different neurotrophic factors are not compromised. Consistent with this conclusion, the
number of neurons in the cortex and hippocampus is similar in the PDK1<sup>K465E/K465E</sup> and
control mice. PKB is a central regulator of survival and apoptosis in many neuronal
types under many different stimuli. Therefore, the reduced activation of PKB observed
in the PDK1<sup>K465E/K465E</sup> neurons might not be limiting to support the neuroprotective role
of this kinase. Consistent with that, we show that the phosphorylation of some key PKB
substrates that promote neuronal survival, including GSK3 and FOXO1, was not
impaired in the mutant cells. Moreover, inhibiting PKBα and PKBβ, but not PKBγ, by
using the Akti-1/2 compound, did not compromise the neuroprotective actions of BDNF
on cortical neurons deprived from serum, a situation that might resemble that of the
PDK1<sup>K465E/K465E</sup> neurons. Because PKBγ accounts in brain for about 40% of the total
PKB protein, this data suggest that either the PKBγ isoform is responsible for BDNF-
mediated neuronal survival, or that as little as 40% of total PKB activation is sufficient
to support the survival of the cortical neurons. Another scenario would be that other/-s
PDK1-activated AGC kinases different from PKB might as well contribute to the
neuroprotective functions of BDNF. In this regard, an specific role for SGK1 in
mediating survival signals in cerebellar granule cells (11) or for RSK in cortical neurons (12) has been also proposed. Mice expressing specifically in the nervous system the PDK1 L155E mutation, disrupting the PDK1 substrate docking site recognition motif, is being generated by conditional knock-in methodology (56). These mice will help much in elucidating the contribution of the docking-site dependent branch of the PDK1 signaling pathway in promoting neuronal survival.

The observation that binding of PDK1 to phosphoinositides contributes to neuronal differentiation is particularly important, because deficient neuronal morphogenesis might result in reduced ability of neurons to integrate and transmit information within the adult central nervous system, a condition which underlies many neurological and mental conditions in human disease. The differentiation of neuronal precursors onto mature neurons involves several stages that can be recapitulated in vitro using dissociated mice cortical as well as hippocampal neurons (46,57). Here we demonstrated that the ability of cortical and hippocampal PDK1K465E/K465E neurons to differentiate in vitro was markedly impaired. This conclusion was sustained on the observations that in the PDK1K465E/K465E cortical neurons, the length of the neurites was significantly reduced, while the axon formation and growth was diminished in the hippocampal neurons. Interestingly, the treatment of wild type hippocampal cultures with the PKB inhibitor Akti-1/2 or the mTORC1 inhibitor rapamycin mimicked the phenotypes of the PDK1K465E/K465E neurons, thereby suggesting that optimal activation of mTORC1 by PKB is required for the formation and outgrowth of the axon. We also show that the profile of expression of the BRSK isoforms along the in vitro differentiation period was blunted in the PDK1K465E/K465E mice, and that re-expression of BRSKs successfully rescued the axonal defects of the mutant hippocampal cultures. The BRSK kinases are required for the polarization of
cortical and hippocampal neurons (7,8). Activation of BRSK isoforms by their upstream kinase LKB1 plays essential roles in neuronal polarization (8). Regulation of BRSK protein expression by TSC2/mTORC1 also influences neuronal polarity (6). Altogether, our data suggests that binding of PDK1 to phosphoinositides, acting through the PDK1/PKB/TSC2/mTORC1/BRSK pathway, is essential to efficiently transmit the extracellular signals that ultimately would modulate neuronal polarization and axon outgrowth. Mice lacking both BRSK isoforms exhibited immobility and poor responsiveness, and died shortly after birth. Close examination of the central nervous system demonstrated smaller forebrain with thinner cortex and neurons failing to form axons (7). As mentioned before, the PDK1<sup>K465E/K465E</sup> mice are nearly viable and exhibited neither overt phenotype nor gross abnormalities in the architecture of the central nervous system, which exhibited normal cortical layering and connectivity (Fig.10). This is in agreement with the fact that the transient character of the PDK1 K465E mutation may allow the accumulation of sufficient levels of BRSK proteins in the adult tissue. However, it would interesting to explore whether the hypomorphic reduction of PKB/mTORC1 activation, causing delayed and/or reduced onset of BRSK protein synthesis and altered neuronal morphogenesis in the PDK1<sup>K465E/K465E</sup> embryo, would lead to more subtle alterations in the patterning of the central nervous system that could ultimately translate onto abnormal behavioral phenotypes.
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FIGURE LEGENDS:

**Figure 1.** Reduced embryonic brain size in PDK1\textsuperscript{K465E/K465E} mice. (A) The number (n) and proportion (%) of mice of the different genotypes resulting from heterozygous breeding are indicated both at embryonic day 15.5 (E15.5) and at birth (P0). ** indicates that the lower frequency than expected of PDK1\textsuperscript{K465E/K465E} pups is statistically significant ($\chi^2$ Test $p < 0.005$). (B) The mean body weights of the indicated PDK1 WT and PDK1 K465E mice at the indicated age in weeks are shown. The values represent the mean ± SEM with each data point obtained from at least five mice per genotype. Representative pictures of the indicated littermate PDK1 WT and PDK1 K465E embryos (E15.5) or 14 days-old pups (P14) are shown in the right panels. (C) Upper panel, the volume of the brain was measured at E15.5 from histological sections using the Cavalieri method. The data are represented as the mean ± SEM for three different mice per genotype. Bottom panel, representative photographs of brains dissected from E15.5 PDK1 WT and PDK1 K465E embryo littermates; scale bar 1 mm. (D and E) The number of cells (upper panels) and the volume of the cell (bottom panels) were determined from E15.5 dissociated cortical (D) or hippocampal (E) neurons. The data are represented as the mean ± SEM for (D) twenty five wild type and twenty nine mutant embryonic cortical cultures obtained from eighteen independent litters, or (E) eight wild type and seven mutant embryonic hippocampal cultures obtained from six independent litters. * $p < 0.05$ and * $p < 0.005$ compared with wild types as obtained by the Student’s t test.

**Figure 2.** Neuronal survival responses are not impaired in the PDK1\textsuperscript{K465E/K465E} mice. (A) Cortical cells of the indicated genotypes were either sham treated (CONTROL), or deprived from trophic factors in the absence (TD) or presence of 50 ng/ml of BDNF (TD+BDNF) for 24 h. Left panel, representative micrographs of PDK1
WT and PDK1 K465E Hoechst-stained cortical neurons after 24 h of the indicated treatment; arrowheads indicate apoptotic nuclei, scale bar 10 μm; right upper panel, the cell viability was determined with the MTT reduction assay and is expressed as a percentage of the untreated cells; data represents the mean ± SEM for at least twenty independent mice embryos per genotype from seven different litters, with each sample assayed in triplicate. Right bottom panel, the percentage of apoptotic cells was obtained by scoring the number of nuclei exhibiting chromatin fragmentation divided by the total; data represents the mean ± SEM for at least nine independent mice embryos per genotype from four independent litters. (B) Cerebellar granule cells were either sham treated (CONTROL), or deprived from serum and potassium in the absence (TD) or presence of 100 ng/ml of IGF-1 (TD+IGF-1) for 24 h; the cell viability was measured as in (A) from three different mice pups each genotype assayed in quadruplicate. (C) Cortical cell cultures in complete media were treated with the indicated doses of staurosporine for 24 h, and then the cell viability determined as in (A) on samples of at least seven independent embryos per genotype from four independent pregnancies, with each sample assayed in triplicate. (D) Cortical cells of the indicated genotypes were treated as in (A) in the presence of the indicated concentrations of BDNF or IGF-1 for 24 h. The cell viability was determined on samples of at least five independent mice embryos per genotype, with each sample assayed in triplicate. * p < 0.05; ** p < 0.005 as obtained with the Student’s t test between trophic deprivation and controls (A and B), BDNF or IGF-1 stimulation and trophic deprivation (A, B and D), and staurosporine compared to untreated controls (C).

Figure 3. The PKB inhibitor Akti-1/2 does not impair neuronal survival. Cortical cells of the indicated genotypes were cultured in complete media or deprived from serum for 24 h in the absence or presence of 50 ng/ml of BDNF, 1 μM of PI-103 or 1
μM of Akt1-1/2, as depicted. The MTT reduction and the percentage of apoptotic nuclei were determined and represented as the mean ± SEM for at least five independent embryos per genotype from two separate experiments, with each sample assayed in triplicate. As a control of the different treatments, cell lysates from matched PDK1 wild type (WT) and PDK1 mutant (KI) littermate mice were immunoblotted with the indicated antibodies. n.s. not significant; *p < 0.05; **p < 0.005 (Student’s t test) between samples treated with BDNF plus the indicated inhibitor and samples treated with BDNF alone.

**Figure 4. Deficient PKB phosphorylation and activation in the PDK1 \(^{K465E/K465E}\) cortical neurons.** Cortical neurons from two PDK1 WT and two PDK1 K465E mice embryos were cultured for 6 DIV, then serum starved for 4 h and either left unstimulated or stimulated with (A) 50 ng/ml BDNF for the indicated time points or (B) for five minutes with the indicated concentrations of BDNF. Lysates were immunoblotted with the indicated antibodies. A representative western blot out of three independent experiments is shown. (C) The effect of the PDK1 K465E mutation on the phosphorylation of PKB at Thr308 and Ser473 was determined by quantitative western blot on cell extracts from cortical neurons stimulated as in (A). Values are expressed as arbitrary units (A.U) and are presented as the mean ± SEM of protein extracts derived from three independent experiments. *p < 0.05; **p < 0.005 (Student’s t test) compared with wild type extracts.

**Figure 5. Analysis of S6K and RSK isoforms in the PDK1 \(^{K465E/K465E}\) cortical neurons.** Cortical neurons from two PDK1 WT and two PDK1 K465E mice embryos were cultured for 6 DIV, then serum starved for 4 h and either left unstimulated or stimulated with 50 ng/ml of BDNF for the indicated time points. Lysates were
immunoblotted with the indicated antibodies to monitor the activation of S6K (A) and RSK (B). A representative western blot of three independent experiments is shown.

**Figure 6. Analysis of SGK in the PDK1K465E/K465E cortical neurons.** Cortical neurons from two PDK1 WT (A and B) and two PDK1 K465E (A) mice embryos were cultured for 6 DIV, then serum starved for 4 h and either left untreated or (A) stimulated with 50 ng/ml BDNF for the indicated time points, or (B) pre-treated with the SB-216763 (3μM), Akti-1/2 (1μM), PI-103 (1μM) or LiCl (10mM) compounds for 30 min as indicated, and then stimulated with 50 ng/ml of BDNF for 5 min. Lysates were immunoblotted with the indicated antibodies (C) Mouse brain and liver tissue extracts from the indicated genotypes were subjected to immunoblot analysis with the indicated antibodies. Each lane corresponds to a sample derived from a different mouse. (D) Multiple sequence alignment of the sequence flanking the indicated phosphorylation sites in NDRG1, NDRG2, PRAS40 and FOXO1. The positions of the first aminoacid aligned according to the mouse protein sequences are indicated. The black shadow indicates similarity, and the grey shadow conservation in more than 50% of the sequences. The phosphorylated threonine or serine residues are labeled with an asterisk. Flanking residues conserved between NDRG1 and PRAS40, but not NDRG2, are marked with an arrowhead.

**Figure 7. Deficient cortical neuritogenesis in the PDK1K465E/K465E mice.** (A) Representative micrographs of the PDK1 WT and the PDK1 K465E cortical neurons at 3 and 4 days in vitro (DIV). Scale bar 50 μm. (B) The total length of the neurites and (C) the soma diameter were measured on digitally acquired images. Each bar represents the mean ± SEM of 200-300 neurons per embryo and four embryos per genotype. *p < 0.05; **p < 0.005 (Student’s t test) compared to wild type controls.
Figure 8. Deficient hippocampal axon formation and growth in the PDK1K465E/K465E mice (A) Representative micrographs of PDK1 WT and PDK1 K465E hippocampal neurons at 2, 3 and 4 days in vitro (DIV) stained with antibodies against the MAP2 dendrite-specific marker (red) and the Tau-1 axonal-specific marker (green). Scale bar 20 μm. The percentage of polarization (B and D) and the axon elongation (C and E) of the PDK1 WT and the PDK1 K465E hippocampal neurons (B and C) or the wild type cells treated with the indicated inhibitors (D and E) was measured at the indicated time points. Each bar represents the mean ± SEM of 300-500 neurons from five different embryos per condition. *p < 0.05; **p <0.005 as obtained with the Student’s t test compared to controls.

Figure 9. PDK1 promotes hippocampal axonogenesis through the PKB/mTORC1/BRSK pathway. (A) Cortical neurons from PDK1 WT and PDK1 K465E embryos were cultured in complete media for the indicated time points. Lysates were immunoblotted with the indicated antibodies. A representative western blot of two independent experiments is shown. (B) Brain whole protein extracts from PDK1 WT and PDK1 K465E mice of the indicated age were subjected to immunoblot analysis with the indicated antibodies. Each lane corresponds to a sample derived from a different embryo or mouse. (C) Hippocampal neurons from PDK1 wild type (WT) and PDK1 mutant (KI) mice embryos were cultured for four days in the presence (RAP) or absence (CON) of rapamycin. Lysates were immunoblotted with the indicated antibodies. A representative western blot of three independent experiments is shown. (D) PDK1 WT and PDK1 K465E hippocampal cells were transfected with the indicated expression vectors at DIV2, and the axon length determined at DIV4. Each bar represents the mean ± SEM of 100-200 neurons from three different embryos per condition. **p <0.005 as obtained with the Student’s t test compared to controls.
Figure 10. Normal layering and connectivity in the PDK1<sup>K465E/K465E</sup> adult brain.

Upper panels, 5 μm-thick coronal brains sections of PDK1 WT (A, C) and PDK1 K465E (I, K) mice stained with hematoxylin & eosin showing the overall architecture of the rostral adult brain. Bottom panels, epifluorescence microscopy images of the PDK1 WT (D-H) and PDK1 K465E (L-P) cortex stained with the dendrite-specific marker MAP2 (D, L), the axonal-general marker SMI 312 (E, M) and the nuclear Hoechst dye (F, N). The merged signals (G, O) as well as adjacent hematoxylin & eosin stained sections (H, P) are also shown. Cortical layers are indicated on the right from I to VI.
Figure 1
Figure 2

A. Images showing the effect of PDK1 WT and PDK1 K465E on cell morphology and MTT reduction. The images are labeled as CONTROL, TD, and TD+BDNF. The graphs to the right show the percentage of MTT reduction and apoptotic cells for each condition.

B. Bar graph showing the MTT reduction in Cerebellar Granule Cells treated with PDK1 WT and PDK1 K465E under control, TD, and TD+IGF-1 conditions. The difference is significant at the 0.01 level (***).

C. Bar graph illustrating the MTT reduction in response to STAurosporine at concentrations of 0, 10, 50, and 500 nM. The bars represent PDK1 WT and PDK1 K465E, showing a significant difference at 0.01 level (***).

D. Bar graph depicting the MTT reduction in responses to varying concentrations of BDNF and IGF-1. The concentrations are 10, 50, and 100 ng/ml for TD+BDNF and TD+IGF-1. The difference is significant at the 0.05 level (*).
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

A. Images of neurons at different DIV (Days In Vitro) stages for PDK1 WT and PDK1 K465E conditions. The images show the distribution of MAP2 (purple) and Tau (green) proteins.

B. Bar graph showing the polarity (%) of neurons with single axons, no axons, and multiple axons for PDK1 WT and PDK1 K465E conditions at DIV 2, 3, and 4.

C. Bar graph showing the axon length (µm) for PDK1 WT and PDK1 K465E conditions at DIV 2, 3, and 4.

D. Similar to B, but for different conditions: control, Akti-1/2, and RAPAMYCIN.

E. Similar to C, but for different conditions: control, Akti-1/2, and RAPAMYCIN.
Figure 10