Excitotoxic inactivation of constitutive oxidative stress detoxification pathway in neurons can be rescued by PKD1

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Excitotoxicity, a critical process in neurodegeneration, induces oxidative stress and neuronal death through mechanisms largely unknown. Since oxidative stress activates protein kinase D1 (PKD1) in tumor cells, we investigated the effect of excitotoxicity on neuronal PKD1 activity. Unexpectedly, we find that excitotoxicity provokes an early inactivation of PKD1 through a dephosphorylation-dependent mechanism mediated by protein phosphatase-1 (PP1) and dual specificity phosphatase-1 (DUSP1). This step turns off the IKK/NF-κB/SOD2 antioxidant pathway. Neuronal PKD1 inactivation by pharmacological inhibition or lentiviral silencing in vitro, or by genetic inactivation in neurons in vivo, strongly enhances excitotoxic neuronal death. In contrast, expression of an active dephosphorylation-resistant PKD1 mutant potentiates the IKK/NF-κB/SOD2 oxidative stress detoxification pathway and confers neuroprotection from in vitro and in vivo excitotoxicity. Our results indicate that PKD1 inactivation underlies excitotoxicity-induced neuronal death and suggest that PKD1 inactivation may be critical for the accumulation of oxidation-induced neuronal damage during aging and in neurodegenerative disorders.

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Neuronal death by excitotoxicity is a critical process in numerous human neuropathologies, such as stroke, traumatic brain injury, epilepsy, Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, amyotrophic lateral sclerosis, and multiple sclerosis. Therefore, intervening the mechanistic steps that lead to excitotoxicity may protect the brain in a broad range of acute and chronic central nervous system pathologies.

Excitotoxicity originates by massive release of the excitatory neurotransmitter glutamate. Overstimulation of postsynaptic glutamate receptors, including the ionotropic N-methyl-D-aspartate (NMDA), α-aminooxy-5-hydroxy-4-isoxazole propionic acid (AMPA), and kainic acid (KA) receptors, overexcites neurons and triggers pro-death cascades.1–3. Primarily, excessive influx of calcium ions occurs, followed by endoplasmic reticulum stress, mitochondrial dysfunction, generation of high levels of reactive oxygen species (ROS), and oxidative stress damage, leading to neuronal death.4–6.

Protein kinase D1 (PKD1), together with PKD2 and PKD3, constitute a family classified within the calcium/calmodulin-dependent protein kinase superfamily. Numerous stimuli activate PKD through well-established pathways. In many cases this activation is transient, but the mechanisms stopping sustained stimulation remain unexplored.7. Oxidative stress is an important activator of PKD1 in cellular models, but its capacity to activate this kinase in vivo is largely unknown.

Excitotoxic production of ROS elevates death-associated protein kinase (DAPK) activity, which provokes neuronal apoptosis in cerebral ischemia and seizure models.8. Accordingly, DAPK absence protects neurons from excitotoxic insults.9, 10. Of note, DAPK can activate PKD in HeLa cells under oxidative stress conditions,11 thus suggesting that PKD activation may contribute to cellular death.

In addition, oxidative stress can also elicit PKD1 activation, determined by Ser916 autophosphorylation, through initial phosphorylation by Abl and Src kinases (at Tyr469 and Tyr93, respectively) and subsequent phosphorylation by protein kinase C delta (PKCδ) of Ser744 and Ser748 (reviewed in ref. 12, see Scheme in Fig. 1a). This cascade promotes cellular survival in non-neuronal tumor cells, through activation of I kappaB kinase (IKK) and nuclear factor-kappaB (NF-κB) that induces SOD2 transcription, a gene encoding the mitochondrial manganese-dependent superoxide dismutase (MnSOD) involved in ROS detoxification.13–17. However, the contribution of NF-κB to neuronal physiopathology is highly controversial, being associated to both neuroprotection and neurotoxicity.18. NF-κB can regulate genes involved either in neuronal survival or in death,19 and there is also some evidence of NF-κB activation by ROS and excitotoxicity in cultured primary neurons.20–22.

To date, to our knowledge there are no studies investigating PKD1 activation by oxidative stress in neurodegeneration animal models or in samples from human disease.

Whether excitotoxic oxidative stress produces PKD1 activation in neurons, and whether this step leads to changes in neuronal NF-κB activity is an important question that remains unanswered. Moreover, the molecular mechanisms involved in PKD inactivation also remain unknown and the contribution of this inactivation to pathophysiological processes has not been investigated. Here we show the existence of a constitutive neuronal PKD1/IKK/NF-κB/SOD2 oxidative stress detoxification pathway that is inactivated by phosphatase-dependent mechanisms during excitotoxic neurodegeneration. Our study demonstrates that PKD1 potentiates neuronal survival by helping neurons to fight against oxidative stress through IKK and NF-κB.

Results

Excitotoxicity regulates neuronal PKD activity. Excitotoxic concentrations of the NMDA receptor (NMDAR) agonist NMDA together with its co-agonist glycine induce neuronal death.23–25. To investigate whether PKD is activated by excitotoxicity, we stimulated cultured primary mature cortical neurons with NMDA (50 μM) and glycine (10 μM), a treatment referred here as “NMDA”, for different time periods and assessed Ser916 autophosphorylation by inmunoblot26 (Fig. 1a, b). PKD basal activity increased 5 min after NMDA addition (Fig. 1b). Strikingly, 30 min and 1 h of treatment decreased p-Ser916 signal markedly below that in control cells (Fig. 1b), indicating a rapid inactivation of PKD. Note that p-Ser916 band appeared as a doublet in unstimulated neurons and that NMDA modified the intensity of both bands (Fig. 1b). Lentinival transduction of PKD1 or PKD2-specific short hairpin RNA (shRNAs) indicated that the upper and lower bands corresponded to PKD1 and PKD2, respectively, and that the PKD antibody detected mainly PKD1 (Supplementary Fig. 1a). In addition, studies by RT-qPCR showed that PKD1 transcripts were more abundant than those for PKD2 and PKD3 in mature cultured neurons, and that excitotoxicity did not affect their levels (Supplementary Fig. 1b, c) or those of total PKD protein (Fig. 1b), suggesting that the observed results may reflect changes in kinases and phosphatases (PPs) activities rather than PKD degradation.

Importantly, non-excitotoxic doses of NMDA (≤10μM) failed to alter PKD activity (Supplementary Fig. 1d). Excitotoxicity was confirmed by the processing of full-length (FL) brain Spectrin to breakdown products (BDPs) by calpain, a protease activated through NMDARs overstimulation27 (Fig. 1b), as well as measuring neuronal viability (Supplementary Fig. 1e). This neuronal death was not blocked by the caspase inhibitor zVAD, suggesting its non-apoptotic nature (Supplementary Fig. 1f). In addition, we detected an increase in ROS production after 1 h of NMDA addition, when PKD appears inactive (Supplementary Fig. 1g), indicating that excitotoxicity-induced ROS production is not paralleled by sustained activation of PKD.

Excitotoxic neuronal death is coupled to stimulation of extrasynaptic NMDARs28 where GluN2B is the major subunit.29 To investigate whether GluN2B is required for NMDA-mediated regulation of PKD activity, we used a selective antagonist for this subunit, ifenprodil (IFN).30 This inhibitor prevented activation (Fig. 1c) and inactivation (Fig. 1d) of PKD. The generic NMDAR antagonist DL-AP5 also blocked the regulation of PKD activity by NMDA (Fig. 1c, d). Notably, DL-AP5 but not IFN increased PKD basal activity (Fig. 1c, d), suggesting that activity of synaptic NMDARs, likely through GluN2A, may contribute to PKD dephosphorylation. As expected, both antagonists hampered the excitotoxic process, as shown by the absence of Spectrin BDPs after 1 h of NMDA (Fig. 1d).

Overactivation of NMDARs triggers a massive Ca2+ influx. Buffering of extracellular Ca2+ with EGTA or treating neurons with the Ca2+ ionophore A23187 demonstrated that the entry of this ion was evoking PKD activity changes (Supplementary Fig. 1h, i).

In conclusion, excitotoxicity drives a short activation followed by a sharp inactivation of PKD that depends on overstimulation of NMDARs containing GluN2B subunits through Ca2+ entry.

Phosphorylation-dependent excitotoxic regulation of PKD. To determine the pathway of PKD activation, we analyzed PKC-dependent activatory transphosphorylation of Ser744/Ser748, and found it changed significantly showing a time-course of phosphorylation/dephosphorylation similar to that showed by p-Ser916 (Fig. 1a, b; Supplementary Fig. 1j). In contrast to the
transient PKD activation, excitotoxicity produced a rapid and sustained activation of DAPK along NMDA treatment, as shown by its gradual activatory dephosphorylation on Ser308 (ref. 9, 10) (Fig. 1b). Together, these results suggest that PKC, rather than DAPK, is the kinase governing PKD activation changes in excitotoxicity.

Under oxidative stress conditions, PKD stimulation mediated by PKCδ phosphorylation is facilitated through Src family tyrosine kinases (reviewed in ref. 12), where Tyr469 and Tyr93 are phosphorylated by Abl and Src, respectively. To study PKD Src-dependent tyrosine phosphorylation, we generated a novel phosphospecific monoclonal antibody (mAb) targeting phosphorylated Tyr93 (see details in Methods and Supplementary Fig. 2).
This antibody detected the upper PKD1 band in neuronal pTyr93 immunoprecipitates and its signal followed a similar pattern of phosphorylation/dephosphorylation as that for p-Ser916 following NMDA addition in the precipitated immunocomplexes (Fig. 1e). It is important to note that pTyr93 signal (and also that of pTyr469) in neurons was strongly potentiated by tyrosine phosphatase inhibitors (Supplementary Fig. 2a). These data suggest that tyrosine PPs might be highly active in mature neurons and contribute to the low level of detection of tyrosine phosphorylated residues unless potent phosphatase inhibitors are used.

Next, we analyzed the effect of the general inhibitors of PKC and Src, GF109203X (GF1) and PP2, respectively, finding that both blocked excitotoxic PKD activation (Supplementary Fig. 3a). Furthermore, rottlerin and SU6656, selective PKCδ and Src family kinase inhibitors, showed the same effect (Fig. 1f, see quantification in Supplementary Fig. 3b), indicating the specific participation of both kinases on the early PKD activation induced by NMDA.

While several kinases involved in PKD activation have been identified, the PPs mediating its inactivation remain unknown.7 We investigated how pharmacological inhibition of different PPs could prevent excitotoxicity-induced PKD dephosphorylation. We pretreated neurons with high concentrations of the serine/threonine PPs inhibitor okadaic acid (OA, 50 nM) to inhibit PKP1 and PP2A activities31 before overstimulating NMDARs. PKD was maintained highly active in the presence of OA under basal conditions or after NMDA addition (Fig. 1g). However, low concentrations of OA (1 nM), able to block PP2A but not PKP131, or PP2B inhibitors did not prevent PKD inactivation (Supplementary Fig. 3c, d). Treatment with the tyrosine PPs inhibitor pervanadate (PV) also increased basal PKD activity and preserved its activation after 1 h of NMDARs overstimulation (Fig. 1g). The PV-induced increase at the higher molecular weight band of p-Ser916 doublet suggested the presence of PKD1-tyrosine-hyperphosphorylated forms in this band (Fig. 1g, solid arrow head). Together, these results suggest a critical involvement of PKP1 and tyrosine PPs in the regulation of neuronal PKD basal activity and in its inactivation during excitotoxicity. They also indicate that activity of PKD in mature neurons might undergo dynamic cycles of phosphorylation/dephosphorylation events due to synaptic activity, likely with the participation of GluN2A subunits and calcium. This fine dynamic synaptic modulation would explain the substantial increases in PKD phosphorylation and activity when all NMDARs or the PPs involved are blocked.

DAPK was dephosphorylated earlier than PKD (Fig. 1b), suggesting that they are regulated by different PPs. However, the kinetics of PKD phosphorylation paralleled those of the mitogen activated protein kinase p38 (Supplementary Fig. 3e and refs. 32–34) suggesting both could be inactivated by the same PPs. The dual specificity phosphatase-1 (DUSP1) and the brain specific striatal-enriched protein tyrosine phosphatase (STEP) are known p38 PPs activated during excitotoxicity and cerebral ischemia35–39. We therefore investigated DUSP1 and STEP contribution to excitotoxic PKD inactivation. STEP dephosphorylation leads to its activation detected by a mobility shift from 61 kDa (STEPα) toward faster-migrating forms36, 37. NMDAR overstimulation increased DUSP1 levels and STEP activation in neurons, both changes paralleling PKD and p38 inactivation (Supplementary Fig. 3e).

Since PKP1 can activate STEP downstream NMDARs overactivation36, 40, 41, we analyzed STEP activity in the presence of OA. We observed that only PKP-inhibitory concentrations of OA blocked STEP activation and led to the accumulation of p-p38 (Fig. 1g, Supplementary Fig. 3c). PV treatment was also effective blocking STEP activation and p38 dephosphorylation (Fig. 1g). In addition, OA and PV decreased DUSP1 excitotoxic induction (Fig. 1g).

Finally, we transduced neurons with lentiviral particles encoding a control shRNA (shC) or shRNA specific for either Dusp1 (shDUSP1) or Step (shSTEP) silencing. Dusp1 knockdown significantly hampered PKD excitotoxic dephosphorylation while Step silencing had no effect (Fig. 1h, i). Of note, shDUSP1 exerted a very similar action to that of PV, increasing the upper band of p-Ser916 doublet, suggestive of DUSP1 acting on p-Tyr residues within active PKD1 (Fig. 1h). Furthermore, Dusp1 but not Step knockdown (Fig. 1h), as PV treatment (Fig. 1g), favored the appearance of a p-p38 higher molecular weight band indicative of p38 hyperphosphorylation, indicating a similar regulation of PKD and p38 by DUSP1.

Ischemic stroke causes neuronal inactivation of PKD. To investigate neuronal PKD inactivation in vivo excitotoxicity, we examined samples from cerebral ischemia. First we used a mouse model of transient cerebral ischemia that consisted in the occlusion of the middle cerebral artery (MCAO) for 1 h followed by 24 h reperfusion42. Hypochromatic Nissl staining marked neuronal injury in the ischemic brain, distinguishing the ischemic core at the striatum (Fig. 2a). Consistent with our in vitro results, immunofluorescence analyses of brain sections showed perinuclear and nuclear dotted staining of active PKD in neurons (NeuN+) in the striatum from sham-operated mice that was absent in the ischemic core at the equivalent region from MCAO-operated animals (Fig. 2b).

**Fig. 1** PKD activity regulation in an in vitro model of NMDA-induced excitotoxicity. a Scheme showing activatory and autophosphorylation sites and domains in PKD1. b p-PKD(S916), p-PKD(S744/S748), PKD, p-DAPK(S308), DAPK, and Spectrin immunoblot analysis of primary mature cortical neurons stimulated with NMDA (50 µM) plus glycine (10 µM) (referred hereafter as NMDA) for various periods of time. Spectrin full-length (FL) and calpain-breakdown products (BDPs) are shown. (Right panel) Quantification of immunoblot signals of p-PKD(S916) relative to total PKD and the loading control neural-specific enolase (NSE). Each time point, p-PKD(S916) value was represented as fold increase relative to control untreated cultures (n = 5 independent experiments). c, d PKD, p-PKD(S916), and Spectrin immunoblot analysis of neurons pretreated for 1h with the GluN2B-specific inhibitor ifenprodil (IFN; 10 µM) or the NMDAR antagonist DL-AP5 (200 µM), and stimulated with NMDA for 5 min (c) or 1 h (d) (n = 3 independent experiments). e Neurons were stimulated with NMDA as above for 5 min or 1 h. PKD1-Y93 phosphorylation after 5 min of NMDA treatment was detected following immunoprecipitation and immunoblotting with a novel phosphospecific monoclonal antibody. Short and long exposure images of p-PKD(S916) and PKD are included. f PKD, p-PKD(S916), and NSE (loading control) immunoblot analysis of neurons pre-incubated for 1 h with the PKC-δ inhibitor Rottlerin (5 µM) or the Src inhibitor SU6656 (5 µM) and treated with NMDA for 5 min (n = 3 independent experiments). g Neurons were exposed for 1 h to okadaic acid (OA; 500 nM), to inhibit serine/threonine phosphatase PPI, or pervanadate (PV; 1 mM), to inhibit tyrosine phosphatases, and then stimulated with NMDA for 1 h. Levels, processing or phosphorylation of PKD, p38, STEP, or DUSP1 were determined by immunoblot analysis (n = 3 independent experiments). h Neurons transfected with lentivirus encoding shC, shDUSP1, or shSTEP were treated with NMDA as indicated. Dusp1 or Step silencing and their effect on PKD inactivation in response to excitotoxicity was analyzed by immunoblotting. i Quantification of immunoblot signal of p-PKD(S916) higher molecular weight band in h relative to total PKD and NSE, represented as fold increase relative to untreated cultures transfused with shC is shown as mean ± s.e.m. (n = 3 independent experiments). *P < 0.05, **P < 0.01; n.s. not significant. two-tailed unpaired Student’s t test. b-h Representative immunoblots are shown.
of neurons had lost p-Ser916 signal compared to the same cortical region in a sham-operated brain (Fig. 2c). This result was reproduced in animals after MCAO for 1 h and a shorter post-ischemic time of 5 h (Supplementary Fig. 4). We further examined the potential relevance of PKD inactivation in human brain tissue from control donors and ischemic stroke patients (Supplementary Table 1). While NeuN+ cells in control cortices showed p-Ser916 immunostaining, cortices of stroke patients lacked active PKD (Fig. 2d).
A critical role for PKD1 in neuronal survival. Next, we investigated whether PKD inhibition could mediate neuronal death. A dose–response and a time-course treatment of cortical neurons with the PKD-specific pharmacological inhibitor CRT0066101 (CRT) showed that 10 μM CRT decreased PKD basal activity and abrogated NMDA-induced activatory response (Supplementary Fig. 5a–c). To examine the effect of this inhibitor on neuronal death, we treated cortical cultures with CRT alone or in combination with NMDA for 4 h and quantified neurons (MAP2+) bearing condensed nuclei as marker of neuronal damage. CRT alone elevated the number of neurons showing nuclear condensation and increased NMDA-induced nuclear condensation (Fig. 3a). MTT viability assays showed similar results, where preincubation with CRT decreased neuronal survival both in basal and excitotoxic conditions (77 ± 6.2% vs. 100% of viability in control cells and 36 ± 4.8% vs. 48 ± 1.9% viable neurons in NMDA-treated cultures) (Fig. 3b). No major changes were obtained at lower CRT doses, except for a small decrease in basal neuronal survival at 5 μM (Supplementary Fig. 5d).

To complete these studies, we transduced primary cortical neurons with lentiviral particles encoding two Prkd1-specific shRNA (shPKD1a and shPKD1b). Once confirmed PKD1 silencing (Fig. 3c), we counted living MAP+ neurons and found that Prkd1 knockdown decreased nearly 70% the number of viable neurons (Fig. 3d). MTT assays showed that interference of Prkd1 decreased neuronal viability in basal conditions and after NMDA-induced excitotoxicity (Fig. 3e).

We next investigated the contribution of PKD1 to neuronal survival in vivo. We generated mice with specific deletion of Prkd1 in CaMKIIα-expressing neurons (PKD1 KO) by crossing Prkd1flloxed mice45 with mice expressing Cre under neuronal CaMKIIα promoter (CaMKIIα-Cre mice) (Supplementary Fig. 6a). We confirmed Cre expression, efficient recombination in the Prkd1 locus and specific decrease of Prkd1 messenger RNA (mRNA) in the cerebral cortex of these mice (Supplementary Fig. 6b, c). Primary cortical neurons cultured from PKD1 KO mice showed lack of PKD1 protein compared to their control PKD1flloxed littermates (Supplementary Fig. 6d). DAB immunohistochemistry of PKD1 KO cerebral cortex showed almost a complete disappearance of PKD antibody specific signal and a substantial decrease in p-Ser916 content (Fig. 3f). Together with results from PKD1 shRNA and immunoblot analysis with these antibodies (Supplementary Fig. 1a), these data strongly suggest that the remaining p-Ser916 signal might correspond to PKD2. Nissl staining, as well as NeuN immunofluorescence did not show noticeable differences between PKD1flloxed and PKD1 KO animals (Supplementary Fig. 6e, f). In addition, these animals did not present macroscopic differences in their cerebrovascular anatomy (Supplementary Fig. 6g). However, after experimental stroke by MCAO, PKD1 KO suffered increased neuronal injury compared to PKD1flloxed animals, as determined by Nissl staining and T2-weighted magnetic resonance imaging (MRI) (Fig. 3g). Our results suggest that although there is no neuronal loss in PKD1 KO mice, their neurons are more sensitive to excitotoxicity and oxidative stress damage. To test this notion, we first determined ROS levels in PKD1 KO vs. PKD1flloxed cultured cortical neurons untreated or treated with NMDA (Fig. 3h; Supplementary Fig. 6h). Although confocal microscopy images evidenced slight ROS increases in untreated PKD1 KO neurons (Supplementary Fig. 6h), flow cytometry quantification analysis showed that differences between the two genotypes only reached significance under excitotoxic conditions, where PKD1 KO contained higher ROS amounts (Fig. 3h). We used MDA, a marker for lipid oxidation by oxidative stress, to analyze the effects of oxidative stress in PKD1flloxed and PKD1 KO brain after sham or MCAO surgery. Importantly, immunofluorescence and quantitative analyses of brain sections showed substantially higher MDA staining in neurons (NeuN+) of sham-operated PKD1 KO compared to PKD1flloxed mice (Fig. 3i). Cerebral ischemia increased MDA labeling in both genotypes but levels in PKD1 KO registered a robust three-fold increase over those in PKD1flloxed brain (Fig. 3i).

**Regulation of IKK/NF-κB pathway during excitotoxicity.** The inactivation of PKD by excitotoxicity led us to hypothesize that the PKD1/IKK/NF-κB pathway may be constitutively active in neurons and that NMDA-induced excitotoxicity and neuronal death could be the consequence of the shut-off of this signaling cascade. The NF-κB transcription factor, formed in neurons mainly by the p65–p50 dimer45, is sequestered in the cytosol through its binding to IκBα, which undergoes proteasomal degradation after IKK phosphorylation46. Therefore, fluctuations in IKK activity are usually translated into changes in the nuclear-cytoplasmic localization and transcriptional activity of NF-κB46. To challenge our hypothesis, we analyzed IKK activity during excitotoxicity finding a decrease in p-Ser176/177 signal indicative of its inactivation after 1 h of NMDAR overstimulation (Fig. 4a). Longer incubations with NMDA produced a stronger decrease in p-IKK levels paralleled by an increase in IκBα (Supplementary Fig. 7a).

We examined the localization of NF-κB p65 (referred as NF-κB) in cultured neurons observing that nearly 100% of MAP2+ neurons presented NF-κB in the nucleus (Fig. 4b) and that NMDA treatment induced significant decreases in NF-κB nuclear signal (Fig. 4b, quantification in the right panel), NF-κB activity was also explored detecting its Ser536 phosphorylation by immunofluorescence. Phosphorylated NF-κB signal constitutively localized at neuronal nuclei and underwent a strong loss after 2 h of NMDA addition (Supplementary Fig. 7b).

To further confirm excitotoxic changes on NF-κB transcriptional activity in neurons, we performed luciferase assays and observed a significant luciferase activity decrease in NMDA-treated neurons (Fig. 4c). Finally, using RT-qPCR we also

### Fig. 2
Neuronal inactivation of PKD in mouse and human ischemic brain. a–c Wild-type mice were sham-operated or subjected to 60 min of MCAO and killed 24 h after reperfusion. (a, left panels) Representative images of cortical brain sections stained with Nissl are shown. The ischemic core in the striatum (S) and in the adjacent cortex (C) corresponding to the penumbra area and the equivalent areas in sham-operated animal are depicted. b, c Representative confocal microscopy images showing predominant localization of p-PKD(S916) staining in NeuN+ cells in brain from sham-operated animals, the absence of active kinase, and NeuN staining at the striatum (b) or the decrease in p-PKD(S916) signal on NeuN+ cells at the cortical penumbar area in ischemic brain (c, left panels). (c, right panel) Percentage of NeuN+ cells containing p-PKD(S916) staining in penumbar zone of MCAO-operated mice compared to the equivalent cortical region of sham-operated animals (n = 100 neurons; n = 3 sections per animal, n = 3 animals per condition). d Decreased number of neurons with p-PKD(S916) staining in postmortem human ischemic stroke samples compared to that from control subjects. Representative confocal microscopy images showing predominant localization of p-PKD(S916) staining in NeuN+ cells in samples C-1 and S-2 are shown (Supplementary Table 1). (Right panel) Percentage of neurons bearing active p-PKD (n = 30–50 neurons per section; n = 3 individuals per condition). e, d Zoom images from boxed regions are also shown. For quantifications, mean ± s.e.m. were derived from the indicated number of samples and analyzed with two-tailed unpaired Student’s t test. ***P < 0.001
determined that mRNA levels of Sod2 and those of Bdnf, a prosurvival neurotrophin whose transcription can be regulated by NF-κB, were downregulated by excitotoxicity (Fig. 4d). Together, these data indicate that neurons present a substantial basal NF-κB activity that is downregulated under excitotoxic conditions.

To determine the relevance of this pathway in vivo, we examined neuronal nuclear NF-κB localization in MCAO-operated mice. According to our in vitro data, NF-κB was present in the nucleus of almost all NeuN+ cells in the striatum and cortex from sham-operated mice. However, ischemia provoked a massive neuronal NF-κB nuclear depletion in the
PKD1 protects neurons through the IKK/NF-κB/SOD2 pathway. Next we assessed the effect of a dephosphorylation-resistant and constitutively active PKD1 mutant in neuroprotection from excitotoxicity. To engineer this mutant, we substituted the four critical residues involved in PKD activation (Tyr93, Tyr469, Ser744, and Ser748) (Fig. 1a) by glutamic acid, to mimic their phosphorylation state. In vitro kinase assays confirmed its constitutive activity (Supplementary Fig. 8a). We fused this quadruple mutant to GFP in a lentiviral vector (PKD1-Ca) bearing human synapsin neurospecific promoter (SYNpr) (Fig. 5a). GFP alone expressed under the same promoter was used as control lentivirus. Neurons transduced with PKD1-Ca lentivirus expressed high amounts of active PKD1 (Fig. 5b) and presented substantial increases in IKK activity (Fig. 5b) and a highly significant induction of SOD2 levels relative to GFP-transduced cells (Fig. 5b). Accordingly, NMDA-induced ROS production in PKD1-Ca neurons was very low (111 ± 10.4%) and similar to that of untreated GFP neurons, compared to NMDA-treated GFP neurons (148 ± 7.8%) (Fig. 5c).

Analysis of MAP2 staining confirmed that PKD1 activation confers neuroprotection against NMDA-induced excitotoxicity (Fig. 5d). Most GFP neurons were not viable after 4 h of NMDA treatment, and presented disorganized MAP2 staining, whereas a prominent number of PKD1-Ca neurons remained viable at this time (Fig. 5d). Importantly, a high number of PKD1-Ca neurons survived even 24 h upon NMDA addition (50.4 ± 10.2%), when almost all GFP neurons were dead (13.5 ± 3%) (Fig. 5d).

Furthermore, NF-κB remained in the nucleus of most PKD1-Ca transduced neurons after 1 h of NMDA treatment in contrast to its cytosolic translocation in a high proportion of GFP neurons (Fig. 5e). The possibility that PKD1-Ca increased neuronal survival by inhibiting Ca2+ influx evoked by NMDARs overactivation was ruled out after performing Ca2+ imaging analysis (Supplementary Fig. 8b).

To demonstrate that increases in SOD2 conferred by PKD1-Ca were dependent on IKK/NF-κB pathway, we treated transduced neurons with the IKK inhibitor SC-514. IKK inhibition decreased SOD2 levels in PKD1-Ca transduced cells (Fig. 5f, left and medium panels). The action of SC-514 was confirmed by the accumulation of IkBα (Fig. 5f, left and right panels). We finally examined the effect that pharmacological inhibition of IKK had on PKD1-mediated neuroprotection. MTT assays showed that IKK inhibition reduced the resistance of PKD1-Ca neurons to excitotoxic death: 98.6 ± 0.6% of PKD1-Ca-transduced neurons survived after 4 h of NMDA treatment, whereas only a 73.79 ± 0.02% of them were alive after combining SC-514 and NMDA treatments (Fig. 5g). Together, our results indicate that PKD1 is neuroprotective against excitotoxic insults by busting the IKK/NF-κB/SOD2 axis and contributing to ROS detoxification.

PKD1 protects against kainic acid-induced excitotoxicity. To investigate PKD1 neuroprotection in vivo, we selected a model of KA-induced neurodegeneration that elicits selective excitotoxic neuronal death particularly in limbic structures (i.e., hippocampal CA1 and CA3 regions) in the rat and mouse brain6.

We first observed in cultured primary neurons that similarly to NMDA, excitotoxic concentrations of KA produced a transient activation of PKD followed by inactivation (Fig. 6a). MTT assays and MAP2 staining showed that KA reduced neuronal viability, and that KA-induced neuronal death was mediated by KA receptors (KARs) (Supplementary Fig. 9a–c). Importantly, KA also provoked the exit of NF-κB from neuronal nuclei (Fig. 6b).

To confirm that PKD and NF-κB were inactivated in neurons during in vivo KA-induced excitotoxicity, we examined the CA1 hippocampal area in control adult rats that received an intraperitoneal injection of saline or KA. CA1 neurons from control saline-injected animals presented active PKD and nuclear NF-κB staining (Fig. 6c). In line with our observations after cerebral ischemia, KA treatment induced condensation of neuronal nuclei in the damaged CA1 area and a marked decrease of NeuN, p-PKD and nuclear NF-κB content in neurons (Fig. 6c), while p-PKD signal emerged in a different cell population. Altogether, these results strongly suggest that excitotoxicity induced by NMDARs or KARs overstimulation triggers similar molecular mechanisms that result in PKD and NF-κB neuronal inactivation preceding their death.
Next we assayed the effects of PKD1-Ca neurospecif
c expression in neuroprotection from KA-induced death. This
was initially performed in vitro, analyzing neuronal cultures
transduced with GFP or PKD1-Ca lentivirus and treated with KA
for 48 h. We found almost complete lack of neuronal death in
PKD1-Ca cultures, in contrast to the extensive neuronal death
provoked by KA in GFP neurons, where nearly a 60% of the
neuronal population died (Fig. 6d).

Finally, to investigate PKD1 neuroprotection in vivo, we
stereotaxically injected GFP or PKD1-Ca lentiviral particles into
the right and left CA1 region of the rat hippocampus, respectively, previous to KA administration (Fig. 7a). In saline-injected animals, GFP+ neurons in CA1 presented nuclear localization of NF-κB independently of whether they were transduced (GFP+) or not (Fig. 7b, see details in zoom images). By contrast, after KA injection there was a clear damage and loss of transduced neurons in the right CA1 GFP-transduced side, as evidenced by sparse fragmented GFP staining and condensation of NeuN-stained nuclei (Fig. 7c). Remaining neurons looked damaged, presented aberrant shape and smaller compacted nuclei, and sparse nuclear NF-κB staining (Fig. 7c). However, neurons in the left CA1 side appeared healthier, not only those transduced with PKD1-Ca (GFP+), but also non-transduced neurons surrounding them, many of which still contained NF-κB in their nuclei (Fig. 7c).

To further support PKD1-mediated neuroprotection using quantitative approaches, we measured hippocampal CA1 neuronal degeneration by Fluorofade B staining. We found a highly significant decrease in KA-induced neuronal death (represented as the % of Fluorofade B staining), in the left CA1 side transduced with PKD1-Ca compared with the right side GFP-transduced (12.65 ± 3% PKD1-Ca vs. 28.19 ± 3.6% GFP) (Fig. 7d).

**Discussion**

Neurons are considered to be highly sensitive to oxidative stress damage, but precisely because they are post-mitotic cells that survive for many years, they must have well developed natural defences against oxidative stress. Here we have discovered that a constitutively active neuronal PKD1/IKK/NF-κB/SOD2 oxidative stress-detoxifying cascade is turned off during excitotoxic neurodegeneration, depleting neurons of this antioxidant natural defence and greatly hampering their survival (Fig. 7e). Our results support the notion that PKD1 enhances IKK activity, NF-κB nuclear localization and SOD2 expression, and decreases ROS production in neurons under oxidative stress conditions.

Excitotoxic damage accumulated along aging or enhanced by acute or chronic neurodegenerative disorders could gradually deteriorate this antioxidant pathway. PKD1 activity withdrawal, accompanied by NF-κB nuclear exit, and subsequent decrease in SOD2 levels would decrease the threshold of the amount of ROS neurons can cope with. ROS elimination through the action of SOD2 would be severely hampered (Fig. 7e). Cultured Prkdl1−/− immortalized fibroblasts showed increased ROS production due to a decrease in the threshold of mitochondrial depolarization, suggesting the possibility that this mechanism may also contribute to increase ROS levels in neurons. The accumulation of ROS in PKD1-deficient neurons, by lack of clearance or by overproduction, may increase oxidative damage and neuronal vulnerability and death. Accordingly, we observed increased MDA labeling in the brain of PKD1 KO mice relative to PKD1+/- littermates.

This PKD–ROS detoxification pathway was identified in a pancreatic cancer mouse model and in cancer cell lines, having as a result the promotion of precancerous lesions and the potentiation of cellular survival, respectively. Contrary to tumor cells, this activation is not sustained in neurons, as demonstrated by decreases in PKD and IKK activities and in NF-κB nuclear localization and activity preceding excitotoxic neuronal death. A transient activation of PKD was found also in dopaminergic cells treated in vitro with hydrogen peroxide, but mechanisms of inactivation or downstream signaling cascades were not explored.

Here, we identify for the first time a molecular mechanism involved in PKD dephosphorylation downstream overstimulation of endogenous NMDARs. This inactivation mechanism depends on PPI and DUSP1, two PP’s whose activities are increased under excitotoxicity. A key point in PKD sustained activation and survival of cancer cells under oxidative stress might rely on their deficiencies in the control of PKD inactivation mechanisms achieved by PPs. For instance, DUSP1 levels inversely correlate with NF-κB activity and their malignancy grade in prostate cancer. In this context, while specific inhibition of PKD1 could provide clear therapeutic advantages in cancer, our results indicate that this type of treatments could represent a high risk for associated neurodegeneration. Conversely, the future development and therapeutic application of PKD activators for neuroprotection should be exquisitely targeted to neurons to unequivocally avoid undesired pro-survival effects on other cell types.

Regarding the existing controversy on NF-κB contribution to neuroprotection and neurotoxicity, our data highlight the importance of this pathway for neuronal survival. This study strengthens the need to perform careful cell-type specific analysis of this and other signaling pathways in complex tissues, identifying pathological changes occurring in cells subpopulations. Revisiting published results obtained from tissue homogenates is essential before drawing final conclusions of the contribution of a specific pathway to disease.

In summary, this study constitutes the first one demonstrating that PKD1 confers neuroprotection against the oxidative stress produced after overstimulation of endogenous glutamate receptors by triggering antioxidant defences and promoting neuronal survival in an excitotoxic environment. On the basis of our results and obtained quantitative data, this study strengthens the need for performing cell-type specific analysis of this and other signaling pathways in complex tissues, identifying pathological changes occurring in cells subpopulations. Revisiting published results obtained from tissue homogenates is essential before drawing final conclusions of the contribution of a specific pathway to disease.
findings, we propose that the development of a broad therapeutic strategy based on preserving PKD1 activity in neurons might be beneficial to slow-down neuronal loss taking place during aging or in a broad range of acute and chronic neurodegenerative diseases by enhancing neuronal natural antioxidant defences.

Methods

Materials and chemicals. NMDA, glycine, cytosine β-D-arabinofuranoside (AraC), poly-L-lysine, α-laminin, IFN, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), Rottlerin, SU6656, EGTA, Z-VAD-FMK, dimethylsulfoxide (DMSO), and sodium orthovanadate (Pervanadate, PV) were from Sigma-Aldrich (St Louis, MO, USA). Bisindolylmaleimide I (GFI), Ca²⁺ ionophore, p-PKD (S916), endog. p-IKK (S176/177), IKK, SOD2, NSE.
AZ2187, and SC-514 were from Calbiochem (UK). Okadaic acid (OA), 3-(4-chlorophenyl)-1-(1,1-dimethyl ethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2), DL-2-amino-5-phosphono pentanoic acid (DL-AP5), and 6-cyano-7-nitroquinoxaline-2, 3-dione (CNSQX) were from Tocris Bioscience (Bristol, UK). Cyclosporine A (CsA) and FK-506 were from Sandoz (Vienna, Austria) and LC Laboratories (Woburn, MA, USA), respectively. KA was from Abcam (Cambridge, UK). CkT0066101 was kindly provided by Cancer Research UK Transfer Technology Department (London, UK).

**Commerical antibodies.** Rabbit polyclonal antibodies were: PKD, phospho-PKD-S916 and phospho-PKD-S744/747, p38 and phospho-p38-T180/Y182, IKKγ, NF-κB phospho-p65-p50 (Cell Signaling Technology, Beverly, MA, USA); phospho-S710/711 IKKβ (Biorbyt, San Francisco, CA, USA); NSE (ICN Biomedicals; Costa Mesa, CA, USA); total and phospho-S580 DAPK (Sigma-Aldrich); Superoxide dismutase (SOD1); DAPK or DAPK substrate-associated protein 2 (MAP2); PKD1-Ca (phospho-Y463) (Abcam); GFP (ThermoFisher Scientific Inventive, Waltham, MA, USA); NF-KB p65, IκB, DUSP1 (Santa Cruz Biotechnology, CA, USA). Mouse monoclonals were: NeuN, Spectrin (Millipore Corporation, Billerica, MA, USA), STEP (Novus Biologicals, Littleton, CO, USA), MDA (Ishac, Japan). Detailed information about all the above-mentioned antibodies and dilutions used for the different applications is given in Supplementary Table 2. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from Santa Cruz Biotechnology and Alexa Fluor 488, -555, and -647 conjugated antibodies were from ThermoFisher Scientific.

**Generation of phosphospecific PKD1 p-Tyr93 monoclonal antibody.** Eight-week-old female BALB/c mice produced at the animal care facility at Centro Nacional de Biotecnología (CNB, CSIC, Madrid, Spain) were immunized subcutaneously with a KLH-conjugated synthetic phosphopeptide corresponding to amino acids 88–98 of human PKD1 (homologous to mouse amino acids 86–96, containing Y93) (KFEPCCGPpYGMRI, where Y refers to acetylamidethyl (Acam) cysteine. Spleen cells from these mice were then fused with P3X63Ag8.653 (ATCC CRL-1580, mycoplasma free) mouse myeloma cells. Hybridoma supernatants were screened for the presence of phosphospecific antibodies by enzyme-linked immunosorbent assay (ELISA) using phosphopeptide and nonphosphopeptide bound to the plate as described previously.25 Selected hybridomas, producing antibodies that reacted specifically with the phosphopeptide, were cloned twice by limiting dilution. We selected a hybridoma whose supernatant reacted specifically with the phosphopeptide and recognized specifically PKD1 phosphorylated at Tyr93 in rat neuronal lysates after peroxidase (PV: 1 mM) or H2O2 (1 mM) stimulation (Supplementary Fig. 2). This antibody was named: Phospho-PKD1 (Y93) monoclonal antibody (clone 6E8). Its isotype was determined by ELISA as IgG2b.

**Experimental animals.** Mice with neuronal conditional deletion of Pckd1 (PKD1 KO mice) in a C57BL/6 background were obtained after crossing Prkd1fl/fl mice in a C57BL/6 background after crossing Prkd1fl/fl mated with CamKIIα-Cre mice (stock number 003539, The Jackson Laboratory, Bar Harbor, ME, USA). Only male PKD1fl/fl and PKD1-KO mice were used and litters were employed in each independent experiment for comparison purposes. Genotyping and recombination analysis in mouse brain cortex was performed by PCR using specific pairs of primers.34 Presence of Cre and flox cassettes was also tested.

**Fig. 5** Phosphatase-resistant active PKD1 enhances IKK/NF-κB/SOD2 pathway and reduces excitotoxicity-induced ROS levels and neuronal death. a Scheme of the lentiviral vector used for neuronal expression of a phosphatase-resistant active PKD1 mutant (PKD1-Ca), where glutamic acids substitute the four activatory residues (Y93, Tyr469, Ser744, and Ser748). GFP alone or fused to PKD1-Ca was cloned under the neurospecific promoter (SYNP). b Immunoblot of PKD and IKK activities and SOD2 levels from primary neurons transduced with GFP or PKD1-Ca lentivirus. Quantitative analysis of medium proteins (p-IKKα and NSE, or (right panel) SOD2) after normalization with NSE, in GFP-transduced neurons (n = 3 independent experiments). c ROS production in GFP- or PKD1-Ca-transduced neurons unstimulated or after NMDARs stimulation for 1 h determined by Cell Rox deep red reagent and FACs analysis, expressed relative to untreated GFP-transduced neurons (n = 4 independent experiments). d Representative images of GFP, MAP2, and DAPI signal of GFP- or PKD1-Ca-transduced neurons treated with NMDA for 4 or 24 h. Zoom-boxed regions are also shown. Transduced neurons were GFP+. (Right panel) Percentage of GFP+ surviving neurons (transduced with GFP or PKD1-Ca) after 24 h NMDA treatment, relative to total GFP+ neurons in untreated conditions (n = 120–250 neurons per condition; n = 4 per group). e Representative images of MAP2, NF-κB, and DAPI staining of GFP- or PKD1-Ca-transduced neurons treated with NMDA for 1 h. See inset with zoom images. (Right panel) Percentage of neurons bearing nuclear NF-κB after or before NMDARs stimulation (n = 50–100 neurons per condition; n = 3 independent experiments). f GFP- or PKD1-Ca-transduced neurons were treated for 6 h with the IKK inhibitor SC-514 (10 μM) or with vehicle (DMSO). Representative immunoblot with the indicated antibodies (left panel) and quantitative analysis of (middle panel) SOD2 and (right panel) IκBα levels normalized to NSE. Data are expressed relative to control GFP+ neurons (n = 4 per group). g Neuronal viability was measured by MTT assays in GFP- or PKD1-Ca-transduced cortical cultures treated for 6 h with SC-514 or vehicle (DMSO) followed by 4 h with NMDA. Data are expressed relative to untreated neurons (triplicates per condition; n = 4 independent experiments). Data are expressed relative to the indicated number of independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant. two-tailed unpaired Student’s t-test.
**Fig. 6** PKD1 mediates neuroprotection from in vitro KA-induced excitotoxicity. 

**a** Primary cortical neurons were stimulated with excitotoxic concentrations of KA (50 μM) for various periods of time and PKD activity and Spectrin processing were determined by immunoblotting. Representative immunoblots are shown (n = 3 independent experiments).

**b** Representative images of MAP2, NF-κB, and DAPI staining of neurons treated with KA for 4 h. Zoom-boxed regions are also shown. (n = 3 independent experiments).

**c** Brain slices from adult male Wistar rats treated with saline or KA (8.5 mg per kg, i.p.) were stained with p-PKD(S916) or NF-κB in combination with NeuN and DAPI. Representative confocal microscopy images of the hippocampal CA1 region are shown (n = 3 animals per conditions).

**d** Representative images of GFP fluorescence and MAP2 and DAPI staining of primary cultured neurons transduced with GFP or PKD1-Ca lentivirus and treated with KA for 48 h. Zoom-boxed regions are also shown. (Right panel) Neuronal survival was determined counting the number of living GFP+ neurons 48 h after KA treatment and shown relative to the number of GFP+ neurons transduced with GFP or PKD1-Ca in untreated conditions (n = 200–400 neurons per condition; n = 3 independent experiments). Data are expressed as mean ± s.e.m. analyzed with two-tailed unpaired Student’s t test. *P < 0.05, ***P < 0.001
micropipettes. A total volume of 2.5 μl per CA1 region of each viral suspension at a concentration of 10⁷–10⁸ pfu per ml was infused at 0.2 μl per min using an automatic infusion pump.

**Treatment with kainic acid.** Twelve days after lentiviral CA1 intracerebral injection, rats were treated with a single intraperitoneal (i.p.) dose of KA (8.5 mg per Kg) or with an equivalent volume of 0.9% saline solution. To verify neurotoxicity induction by KA, behavioral observations were made during 3 h post injection. All KA-treated animal displayed general limbic seizure activity. Seizures were scored as previously described. Three days after KA treatment, animals were anesthetized and perfused for further immunohistochemistry analysis.

**Primary culture and treatment of cortical neurons.** Rat cortical neurons were prepared from cerebral cortex of 19-day-old Wistar rat embryos as previously described. Briefly, cerebral cortices were dissected and mechanically dissociated in 4 ml of MEM culture medium (Eagle’s minimum medium, ThermoFisher Scientific Gibco), supplemented with 5% fetal bovine serum, 5% horse serum, 22.2 mM glucose, 0.1 mM Glutamax-I (ThermoFisher Scientific Gibco), penicillin (100 U per ml) and streptomycin (100 U per ml). Cells were plated at a density of 2.5 × 10⁵ cells per cm² in the same medium in plates or coverslips previously treated with poly-L-lysine (100 μg per ml) and laminin (4 μg per ml). At day 7, cytosine β-D-arabinofuranoside (10 μM) was added to the culture and maintained until the end of experiments to inhibit growth of glial cells. After 14 days in vitro (DIV), neurons were pretreated or treated for different time periods as indicated with the following concentrations of compounds: 50 μM NMDA and 10 μM glycine (combination referred as “NMDA” along the manuscript), 200 μM DL-AP5, 10 μM IFN, 4 μM A23187, 2 mM EGTA, 25 μM Z-VAD-FMK, 20 μM CNQX, 3.5 μM GFL, 5 μM Rottlerin, 5 μM PP2, 5 μM SU6656, 500 nM or 1 nM OA, 1 mM PV, 200 ng per ml FK506, 100 ng per ml CsA, 10 μM CRT, 20 μM SC-514, 50 μM KA, 1 mM H₂O₂. Excitotoxicity was induced in cortical neurons by overstimulation of NMDARs with the co-agonists NMDA and glycine.
or by overstimulation of KARs with KA. Unless otherwise stated, inhibitors were added 1 h before NMDA treatment and remained in the culture media for the duration of the experiment.

 Cultures of mouse cortical neurons from E17 brain cortex of PKD-flxed and PKD-KO animals were prepared as previously described36.Briefly, tissue was incubated in a 0.25% trypsin solution in Ca2+/Mg2+ free Hank's buffered salt solution (HBSS) and dissociated using fire polished Pasteur pipettes. Then neurons were seeded onto poly-L-lysine coated cover slips in 24-well plates with 0.5 × 106 cells per cm2 on coverslips or dishes as above in MEM complemented with 10% FCS, 2 mM Glutamax-I, 100 U/ml penicillin, 100 U/ml streptomycin, 100 U/ml pen for 2 h until they attached. Medium was then replaced by Neurobasal medium containing 2% B27 supplement and 2 mM Glutamax-I (Thermo Fisher Scientific) (0.1 M Cytosine β-2-aminorafuronoside (10 μM) was added to the culture at DIV3. Neurons were maintained in the same original medium and used at DIV11. All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO2.

 Preparation of protein extracts, immunoprecipitation, and immunoblot analysis. Protein extracts from primary cultures were prepared in RIPA as previously described33,34. Briefly, cultured neurons were lysed in RIPA buffer (25 mM Tris-HCl, pH 7.6, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl) with protease and phosphatase inhibitors for 30 min at 4 °C. lysates were centrifuged for 30 min at 14,000 rpm at 4 °C, and resulting supernatant was considered the total lysate soluble fraction. For immunoprecipitation of PKD-1 p-Tyr93, 1 mg of neuronal protein extracts was incubated with 50 μl of the p-Tyr93 hybridoma supernatant. After 4 h at 4 °C, immunocomplexes were bound to protein G Sepharose for 1 h at 4 °C. Beads were washed four times with RIPA buffer before solubilization in SDS-PAGE sample buffer. Equal amounts of immunoprecipitates were resolved in SDS-PAGE and analyzed by immunoblot. Membranes were incubated with different primary and secondary antibodies and immunoreactive bands were detected by ECL (PerkinElmer, Waltham, MA, USA). Immuno blot images have been cropped for presentation. Full size images are presented in Supplementary Figs. 10–15.

 RNA isolation and reverse transcription-PCR and quantitative real-time PCR analysis. Total RNA from the cortex of PKD-flxed and PKD-KO mice was isolated with RNeasy Mini Kit (Qiagen, Germany). The cDNA was synthesized using oligo-dT extension primers from 1 μg RNA premade cDNA, using ProtoScript M-MLV reverse transcriptase (Promega, Madison, WI, USA) and 5 μl of 2× SYBR Green Mastermix (Life Technologies). Lentiviral vectors containing shRNAs to interfere with PKD1 (shDUSP1) or the quadruple mutant generated here with constitutive kinase activity (GP-DUSP1-Ca) or the quadruple mutant generated here with constitutive kinase activity (GP-DUSP1-Ca) for 48 h. Total lysates were immunoprecipitated with anti–phospho-p38 antibodies and PKD1 kinase activity was assessed by in vitro kinase assay as previously described35. Briefly, immunoprecipitates were washed four times with RIPA buffer and twice with kinase buffer (30 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 5 μM diethiothreitol) containing 10 μCi [32P]ATP and incubated for 10 min at 30 °C. The reaction was stopped by adding sample buffer and immunoprecipitates were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Filters were exposed to obtain autoradiography images and then subjected to immunoblot analysis with anti–phospho-p38 antibody.

 Plasmids for shRNA. Lentiviral vectors containing shRNAs to interfere with Dusp1 (shDUSP1) or Step (shSTEP) were generated by cloning the following oligonucleotides into the HpaI and XhoI sites of pLenti3.7 (pL73): shDUSP1 5′-GTAACCACTTT GAGGGTCACTAGAATTCTAGTGACCCTCAAAGTGG-3′, forward 5′-GGACCCTGAGCTTACTCTCTGTTGGAATGATTGCTCCGGAATCTCTCAGGTTT-3′, reverse 5′-GGCCGCCTCGAGGTTTCTGGGATGACGGGCATAAGAGC-3′. The selected vector was amplified using forward (5′-GGCCTACGAGCATTTTCTGGCTGAG-3′) and reverse (5′-GGCTACCAATTTTTGGGAGGACTGGG-3′) primers containing an AvrII and PacI sites, respectively (underlined). Once digested, the PCR product was cloned in a lentiviral vector bearing human Synapsin promoter (SYNpr) Synpr-DsRed-SYNpr, where DsRed was depleted after digestion with BamHI and NotI and substituted by an adapter with multiple cloning sites containing AvrII and PacI sites (5′-GATCCTGCAGCGCGCATGGCTTCCCTTGCACGACTGTTTTTCGTCTTGCCGCAACCCAACAGGTTT-3′ and 5′-TGGCAAAAGGCCCCGGAGATTCACCGGGAGATAGCTTTTCTGTGCTG-3′). The PCR product was amplified using forward (5′-GCGCTACGAGCATTTTCTGGCTGAG-3′) and reverse (5′-GGCTACCAATTTTTGGGAGGACTGGG-3′) primers containing an AvrII and PacI sites, respectively (underlined). The second SYNpr and the GFP cassette were also eliminated by digestion with Shpi and EcoRI, klenow refilling and religation. Constructs were sequenced using an Applied Biosystems automated DNA sequencer (Thermo Fischer Scientific).

 In vitro kinase assays. HEK-293T cells were transfected with p-EF-Bos-GFP empty or containing wild-type PKD1 (GFP-PKD1), a kinase-dead mutant (GFP-PKD1-KD) or the quadruple mutant generated here with constitutive kinase activity (GFP-PKD1-Ca) for 48 h. Total lysates were immunoprecipitated with anti–phospho-p38 antibodies and PKD1 kinase activity was assessed by in vitro kinase assay as previously described35. Briefly, immunoprecipitates were washed four times with RIPA buffer and twice with kinase buffer (30 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 5 μM diethiothreitol) containing 10 μCi [32P]ATP and incubated for 10 min at 30 °C. The reaction was stopped by adding sample buffer and immunoprecipitates were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Filters were exposed to obtain autoradiography images and then subjected to immunoblot analysis with anti–phospho-p38 antibody. The selected vector was amplified using forward (5′-GGCCTACGAGCATTTTCTGGCTGAG-3′) and reverse (5′-GGCTACCAATTTTTGGGAGGACTGGG-3′) primers containing an AvrII and PacI sites, respectively (underlined). The second SYNpr and the GFP cassette were also eliminated by digestion with Shpi and EcoRI, klenow refilling and religation. Constructs were sequenced using an Applied Biosystems automated DNA sequencer (Thermo Fisher Scientific).

 Generation of PKD1 mutant and cloning in a lentiviral vector for neurospecific expression. Mutant PKD1-Y93E/Y469E was generated by overlapping PCR using primary cultures as a DNA template as previously described36. A sequence upstream of PKD1 cDNA corresponding to pBSK and close to the polylinker and a sequence near to the Sp6 site within PKD1 were used as external forward (5′-GACCTAACTTATAGGCCAACCTGGTTAAGCGGTAACCGGAACTCCCGGCTTTTATCTGAGTCTCTGCTCCTCTTGATGAGATCCATGCACATGTTGCTGCCTGTCTGCTTCGGTCAGG-3′) and reverse (5′-CCTGGGATGACCCGACGGGATACACCCGAGGTCGTCCTCCGCCTCCTCTTCTGTTTCATTTGAGGTCGCTGCTCTG-3′) primers together with internal complementary forward (5′-TCCCGGAATGTTGCTTGGGACCTGTTGAGAAGCTGATG-3′) and reverse (5′-GATTTTTACATGAAAGAGAGGACAAGGAGAAAGAGGAGGACGGGCAACCATCCATCAGTCTGCACTG-3′) primers containing the residual substitution for Y93E (bold). After the second PCR reaction, the amplified fragment was digested with Xhol and Spfl, and the wild-type PKD1-Xhol/Spf1 fragment was then replaced. For double mutant PKD1-Y93E/Y469E/EGF, we followed the same approach, using as template pBSK- PKD1-Y93E mutant and internal primers forward (5′-ACAGGAAGACCTGGAACCCGAGGTCGTCCTCCGCCTCCTCTTCTGTTTCATTTGAGGTCGCTGCTCTG-3′) and Y93E substitution (bold). The quadruple mutant PKD1-Y93E/Y469E/EGF/PRD5/PRD6/PRD7 was obtained by digesting pBSK-PKD1-Y93E/EGF/PRD5/PRD6/PRD7 with Xhol and Spfl, and using this fragment to substitute that of pBSK-PKD1-S545E/EGF (a mutant obtained before36). This quadruple mutant was digested with EcoRI and subcloned in p-EF-Bos-GFP as we have previously done36. Then, FL fusion sequence GFP-PKD1-Y93E/EGF/PRD5/PRD6/PRD7 was obtained by digesting pBSK-PKD1-Y93E/EGF/PRD5/PRD6/PRD7 with Spfi and EcoRI, klenow refilling and religation. Constructs were sequenced using an Applied Biosystems automated DNA sequencer (Thermo Fischer Scientific).
Lentiviral production and transduction of neuronal cultures. Lentiviral suspensions were prepared in HEK293T cells as previously described. Briefly, HEK293T cells were transfected with lentiviral vectors and packaging vectors using Lipofectamine 2000 reagent and OPTI-MEM media (ThermoFisher Scientific Gibco) for 4 h following the manufacturer instructions. Medium was then changed to IMDM complemented with 5% fetal bovine serum, 5% horse serum, 100 U per ml penicillin, 100 U per ml streptomycin, and 4% DMSO. Supernatant was collected after 48 h. For concentration, the viral suspension was first filtered using a Steriflip-15 0.45-μm filter unit (Millipore, Billerica, MA, USA) and ultracentrifuged at 20,000 rpm for 2 h at 4°C in a SW28 Beckman Coulter rotor. Viral pellets were resuspended overnight at 4°C in PBS. HEK-293T cell line was purchased from ATCC and tested to be mycoplasma-negative. DIV7 neurons were transduced with concentrated lentiviral suspensions (10–100 pfu per ml) directly added to the growing media for 7 additional days.

Assessment of neuronal viability in neuronal cultures. Analysis of neuronal survival was measured by MTT reduction assay (Sigma-Aldrich), as previously described. Briefly, MTT (0.5 mg per ml) was added to the medium of neuronal cultures, and after 2 h at 37°C, medium was replaced and the formazan salts formed were solubilized in 100 μl of DMSO and spectrophotometrically quantified at 570 nm. Data were represented as the percentage of neuronal viability giving to the control conditions a 100% value. Neuronal death was also analyzed by determining nuclear condensation and neuronal shape, visualized by DAPI and MAP2 staining, respectively, and expressed as percentage of nuclear condensation. Viability of neurons transduced with GFP or PKD1-Ca lentiviral particles was expressed as the percentage of GFP+ neurons after NMDA or KA treatments at 37°C for 30 min.

Determination of ROS production in cultured neurons. Rat cortical neurons DIV4 were treated with NMDA for 1 h and then washed and loaded with 10 μM Cell ROX Deep Red fluorescent probe (ThermoFisher Scientific), by incubating them for 10 min in the dark. The medium was then removed and cells were washed once with PBS, trypsinised and collected. Then, cells were washed twice with PBS and fixed with 4% PFA for 10 min at room temperature. Cellular fluorescence intensity was measured using a FACSCanto II flow cytometer (BD Biosciences, Allschwil, Switzerland). For each analysis, 10,000 events were recorded. ROS production was estimated using the mean fluorescence intensity of each cell population. In neurons transduced with GFP or PKD1-Ca, the mean of the fluorescence intensity relative to the total of GFP+ population was measured.

Luciferase assays. Cortical neurons plated in 24-well plates were transfected at DIV7 with (NF-kB)-tk-Luc or tk-Luc, together with renilla luciferase plasmids. Cells were transfected in serum-free medium by using 0.5 μg of DNA and 0.5 μl of Lipofectamine 2000 reagent (Invitrogen) per well, according to the manufacturer’s specifications. Two days after transfection, neurons were treated with NMDA for 3 h and processed for luciferase activity measurements. Luciferase activity was detected by adding the enzyme substrate provided in the Dual-GloTM luciferase determining kit (Promega Corporation, Madison, WI, USA). It is important to point out that we confirmed that GFP fluorescence was not affecting FluoroJade B staining. For that purpose, we combined FluoroJade B labeling with GFP immunostaining using an anti-GFP antibody detected with secondary Alexa-555 antibodies and found absolutely no co-localization of both signals by confocal microscopy analysis.

FluoroJade B staining of rat brain. To quantify brain damage in KA-treated rats, a different set of coronal brain sections adjacent to that used for immunofluorescence analysis was stained for in situ cell death using FluoroJade B staining (Millipore Corporation, Billerica, MA, USA). It is important to point out that we confirmed that GFP fluorescence was not affecting FluoroJade B staining. For that purpose, we combined FluoroJade B labeling with GFP immunostaining using an anti-GFP antibody detected with secondary Alexa-555 antibody and found absolutely no co-localization of both signals by confocal microscopy analysis. Paraflin-embedded human postmortem stroke brain tissue samples were cut in 15-μm sections, transferred to citrate buffer (pH 6.0) for antigen retrieval, and incubated in 10% donkey serum for 1 h. For double immunofluorescence analysis, sections were incubated with primary antibodies for 2 days in blocking solution followed by washes in PBS and incubation with secondary antibodies in the same solution. Then, slices were rinsed in PBS and nuclei were stained with DAPI. Sections were treated with a saturated solution of Sudan black B (Merck Bioscience, Darmstadt, Germany) for 15 min to block autofluorescence of lipofuscin granules. The sections were later washed and mounted with Prolong medium.
were extensively washed in H₂Oₐ and transferred to a staining solution containing 0.1% acetic acid and 0.0004% FluoroJade B for 30 min in the dark. Slides were again rinsed in H₂Oₐ, dried, and submerged directly into xylene and mounted in DPX medium (Sigma-Aldrich). For quantification analysis, CA1 regions were outlined, and total FluoroJade B staining in the outlined region was measured using a densitometric thresholding technique implemented with ImageJ 1.47d software (NIH). The threshold was set at a level just above that with counted background and non-specific staining in areas outside the outlined region. The analysis was done in a series of sections through the entire hippocampus from each rat brain. Data were represented as the % FluoroJade B staining and indicate the total number of stained pixels over threshold.

**Brain vascular anatomy.** PKD1<sub>flxed</sub> and PKD1-KO mice littersmates brain vasculature was analyzed as described<sup>48</sup>. Briefly, mice were anesthetized by an i.p. injection of pentobarbital and then 2 ml of 5% Evans blue solution in saline was perfused intracardially for 1 min. Animals were killed and brains were fixed by immersing the skulls in 4% PFA for 48 h. Macroscopic images of ventral and dorsal views of the brain were acquired under a stereo microscope (M50, Leica, Germany) under cold light illumination using a digital camera (Infinity 2 Digital Camera, Nikon). Quantification of dorsal surface brain images was performed with ImageJ software, measuring the distance between the anastomotic line, drawn as previously described<sup>22</sup>, and the midline at 3 and 6 mm from the frontal pole of the brain.

**Images acquisition.** Confocal microscopy images were acquired using plan-apochromatic objectives in an inverted Zeiss LSM 710 laser scanning microscope (Zeiss, Germany). To avoid crosstalk between channels, sequential scanning mode was used. All images shown correspond to the maximum intensity projection of serial sections; in case of different projections, details can be found in figure legends. Pictures were processed with Zen 2009 (Carl Zeiss MicroImaging), Adobe Photoshop CS (Adobe Systems Inc.), and ImageJ 1.47d (NIH) software. Nissl and DAB staining bright field images were captured in a Nikon Eclipse 90i microscope, using a Digital Sight DS-QiMc camera, and NIS-Elements BR 3.0 software.

**Quantitative and statistical analysis.** Immunoblot signals were quantified by densitometric analysis (NIH Image) and normalized using NSE. Phospho-antibodies signals were normalized using the total protein values relative to those of NSE. Data were expressed relative to values obtained in their respective untreated controls. Neuronal viability in cortical cultures was measured by MTT or DAPI condensation. Results are shown as mean ± s.e.m. and the number of experiments or outcome assessment. Results are shown as mean ± s.e.m. and the number of experiments carried out with independent primary neuronal cultures and animals (n) is shown in figure legends.

**Data availability.** The data sets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Additional information

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