PLD1 participates in BDNF-induced signalling in cortical neurons

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The brain-derived neurotrophic factor BDNF plays a critical role in neuronal development and the induction of L-LTP at glutamatergic synapses in several brain regions. However, the cellular and molecular mechanisms underlying these BDNF effects have not been firmly established. Using in vitro cultures of cortical neurons from knockout mice for Pld1 and Rsk2, BDNF was observed to induce a rapid RSK2-dependent activation of PLD and to stimulate BDNF ERK1/2-CREB and mTor-S6K signalling pathways, but these effects were greatly reduced in Pld1−/− neurons. Furthermore, phospho-CREB did not accumulate in the nucleus, whereas overexpression of PLD1 amplified the BDNF-dependent nuclear recruitment of phospho-ERK1/2 and phospho-CREB. This BDNF retrograde signalling was prevented in cells silenced for the scaffolding protein PEA15, a protein which complexes with PLD1, ERK1/2, and RSK2 after BDNF treatment. Finally PLD1, ERK1/2, and RSK2 partially colocalized on endosomal structures, suggesting that these proteins are part of the molecular module responsible for BDNF signalling in cortical neurons.

Brain-derived neurotrophic factor (BDNF) is the most widely expressed and well-characterized member of the neurotrophin family in the mammalian brain. It is translated as a precursor protein (proBDNF), consisting of an N-terminal prodomain and a C-terminal mature domain. Mature BDNF is composed of dimers, and its effects are tightly regulated. BDNF can exert its functions in a highly localized manner, and also at a distance by anterograde or retrograde transport. Modest changes in BDNF levels affect the development and regulation of neural circuits and brain function. BDNF has been shown in cell culture to play an important role in neuronal survival and in the maintenance of most neuronal networks and is important for functional and structural synaptic plasticity1–3. The current notion is that during development BDNF is involved in regulating the fine-tuning of the cortical network by selectively enhancing dendritic growth in an activity-dependent manner4,5. Furthermore, dendritic spine density and morphology of mature primary hippocampal neurons are significantly influenced by BDNF6,7.

On the other hand, the in vivo role of BDNF has been very difficult to evaluate in the post-natal brain as Bdnf−/− mice die shortly after birth8. A number of studies using different conditional gene targeted mouse lines and Cre-loxP-mediated excision of Bdnf have led to the conclusion that in vivo the effects of endogenous BDNF in modulating the structure of neurons seem to be extremely specific, depending on the developmental stage, the brain area, as well as the cell-type. Another level of complexity lies in the multiple downstream signalling cascades, as well as the diametrically opposing effects of the pro- and mature forms which act through distinct receptors, tropomyosin-like kinase B (TrkB) and p75 (neurotrophin receptor, NTR) respectively.

The synergistic interactions between neuronal activity and synaptic plasticity induced by BDNF make it an ideal and essential regulator of cellular processes that underlie cognition and other complex

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behaviours. Indeed, numerous studies have firmly established that BDNF plays a critical role in hippocampal long-term potentiation (LTP), a long-term enhancement of synaptic efficacy implicated in learning and memory. Converging evidence now strongly implies that deficits in BDNF signalling contribute to the pathogenesis of several major diseases and disorders, such as Huntington’s disease, Alzheimer’s disease, and depression. Thus, manipulating BDNF pathways represents a viable therapeutic approach for a variety of neurological and psychiatric disorders.

Several recent reports suggest a link between neurotrophins, neuronal development and phospholipase D (PLD) activity. For example PLD1 regulates basic fibroblast growth factor (bFGF)-induced neurotrophin-3 expression and neurite outgrowth in immortalized hippocampal progenitor cells. We have recently shown that cortical neurons cultured from mice lacking Pld1 exhibit a significant delay in growth and development. In line with this observation, Pld1 knockout mice display impaired brain development and reduced cognitive function up to one month of age. Furthermore, we found that neuronal growth factor (NGF)-induced neurite outgrowth requires phosphorylation of PLD1 by the serine-threonine kinase RSK2 in PC12 cells, and that the production of phosphatidic acid facilitates exocytosis of vesicle-associated membrane protein (VAMP)-7 vesicles at growth cones. Interestingly the phosphorylation site for RSK2 is not found in PLD2, suggesting that this pathway is specific for PLD1 signalling. A loss of function mutation in RSK2 is responsible for the Coffin–Lowry syndrome (CLS), a rare syndromic form of mental retardation (MR) that shows X-linked inheritance. These data suggest that the loss of RSK2 leading to CLS and neuronal deficits is related to defects in neuronal growth due to impaired RSK2-dependent PLD1 activity following NGF stimulation. Here, we investigated whether PLD1 is directly involved in BDNF signalling through a process involving RSK2 and found that PLD1 contributes to the regulation of multiple intracellular signalling cascades, including retrograde messages relying on vesicular PEA15 complex.

Material and Methods

**Materials.** Antibodies anti-HA (Babco), anti-RSK2, anti-APPL1, anti-Rab7, anti-TrkB (Santa Cruz Bio-technology), anti-PLD1, anti-ERK, anti-phospho-ERK, (New England BioLabs), anti-β-tubulin, anti-CREB (Millipore), anti-GAPDH, anti-phospho-CREB (Ser-133), anti-mTOR, anti-phospho-mTOR (Ser-2481), anti-phospho-S6K (Thr-389), anti-phospho-S6K (Thr-421/Ser-424), anti-PEA15 (Cell Signalling), anti-Rab5 (Transduction Laboratories) were used. Plasmids have been described previously. ON-TARGETplus siRNA were obtained from Darmacon and BDNF was from Invitrogen.

**PLD assay.** WT and Rsk2<sup>−/−</sup> cortical neurons from E17 mice were plated at 40,000 cells per well and at 3 DIV were incubated for increasing time with 100 ng/mL of BDNF and used to measure PLD activity as described previously. Briefly cells were washed twice with PBS and medium was then replaced by 100 µl of an ice-cold Tris 50 mM pH 8.0 solution and the cells broken by three freeze and thaw cycles. Samples were collected, mixed with an equal amount of the Amplex Red reaction buffer (Amplex Red Phospholipase D assay kit, Molecular Probes, USA) and the PLD activity estimated after 1 h incubation at 37 °C with a Mithras (Berthold) fluorimeter. A standard curve was performed with purified PLD from Streptomyces chromofuscus (Sigma). Data are normalized to the activity in WT neurons in the absence of treatment.

**Animals, Cell Culture and BDNF treatment.** Pld1<sup>−/−</sup> and Rsk2<sup>−/−</sup> mice have been described previously. They were housed and raised at Chronobiotron UMS 3415. All experiments were carried out in accordance with the European Communities Council Directive of 24th November 1986 (86/609/EEC) and resulting French regulations. Accordingly all experimental protocol were approved by the CREMEAS local ethical committee. Every effort was made to minimize the number of animals used and their suffering. Cortical neurons were cultured from E17 mice in Neurobasal medium (Invitrogen) supplemented with B-27, 1% GlutaMAX and 1% Pen/Strep. Low-density cultures were plated on poly-L-lysine (Sigma)-coated glass coverslips at 25000 neurons/cm². BDNF was applied to cells 72 h after transfection at 100 ng/mL for the indicated times.

**Transfection.** Cultured cortical neurons were transfected at 2 DIV with Lipofectamine 2000 (Invitrogen) according to the manufacture’s instruction and analysed between 3 and 6 DIV. Transfection efficiency for HA- and GFP-tagged protein ranged between 15 and 25%.

**Western Blot and Immunoprecipitation.** After treatment, cells were lysed and proteins were resolved by SDS 4–12% PAGE. Proteins were transferred to nitrocellulose membranes as previously described (de Barry et al., 2006). Detection was performed by chemiluminescence using the Super Signal West Dura Extended Duration Substrate (Pierce). For immunoprecipitations, protein extracts were prepared in lysis buffer (50 mM Hapes, 3 mM EGTA, 3 mM CaCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, 80 mM KCl, 0.1% Triton X-100, 0.1% sodium deoxycholate, 1 mM sodium orthovanadate, 40 mM NaF; and protease inhibitor mixture (Sigma-Aldrich)). Five hundred micrograms of total protein were used. Quantification of Western blots was performed as described earlier.
**Immunocytochemistry.** Neurons were fixed and further processed for immunofluorescence as described previously. Stained cells were visualized with a Leica SP5II confocal microscope. Quantification of co-localization was performed using Image J (JACoB plugin). Nuclear levels of phospho-ERK and phospho-CREB staining were estimated by the ratio: fluorescence intensity/the nucleus area, the latter being estimated by DAPI staining. For imaging analysis at least 100 cells were analysed for each condition obtained from at least two independent cell cultures.

**Statistics.** Number of samples, cell analysed and repeats are indicated in the figure legends. Statistical analysis was performed with R software using parametric and non parametric t-tests compared to the corresponding control condition (*p < 0.05; **p < 0.01; ***p < 0.001).

**Results**

**RSK2 is required for neuronal PLD1 activation by BDNF**. Using cultures E17 mouse cortical neurons, we observed that RSK2 and PLD1 are highly expressed during the first week of culture, with expression levels for both proteins being maximal at 6 days in vitro (DIV) (Fig. 1A). Interestingly, formation and development of neuronal dendrites occurred between 4 and 15 DIV, suggesting that the activity of RSK2 and PLD1 could be involved in this process, in agreement with our recent finding that PLD1 KO neurons have less complex arborisation. At earlier time (3DIV) when expression levels of PLD1 are submaximal, BDNF induced a time-dependent increase in PLD activity in cortical neuron cultures with a maximal effect measured after 30 min of stimulation (Fig. 1B). On the other hand, BDNF failed to trigger PLD activity in Rsk2−/− neurons, suggesting that RSK2 may be an essential element in the signalling cascade that leads to BDNF-induced PLD activation.

**PLD1 regulates the ERK1/2-CREB and mTor-S6K signalling pathways in response to BDNF**. BDNF stimulation of cortical neurons induced a rapid phosphorylation of ERK1/2 that reached a maximum near 15 minutes of treatment (Fig. 2A). In the absence of PLD1, the phosphorylation levels of ERK1/2 (p-p42 and p-p44) after BDNF treatment were significantly reduced (Fig. 2A), suggesting that PLD1 plays a positive role in the optimal activation of the ERK signalling pathway in response to BDNF. BDNF also induced a strong increase in phospho-CREB levels (p-CREB), an effect that was almost completely abolished in Pld1−/− neurons (Fig. 2B), in line with the idea that PLD1 activity contributes to the ERK-CREB signalling pathway. At the same time, we found that PLD1 is required for optimal BDNF-induced phosphorylation of mTor (p-mTor) and S6Kinase (both for p-70(389Thr)-S6K and p-70(421Thr/Ser424)-S6K) (Fig. 2B), suggesting that PLD1 also affects this signalling pathways.

**PLD1 overexpression modulates the nuclear level of p-ERK1/2 and p-CREB**. Since BDNF has been reported to affect the nuclear levels of p-ERK1/2 and the transcription factor p-CREB22,23, we next...
Figure 2. PLD1 is involved in different signalling pathway triggered by BDNF in cultured cortical neurons. (A) WT and Pld1⁻/⁻ cortical neurons in culture for 3 DIV were treated for the indicated time with 100 ng/mL of BDNF and lysed. 35 μg of proteins was resolved by SDS-PAGE and probed with anti-ERK1/2, anti-phospho-ERK1/2 and anti-GAPDH antibodies. The phosphorylation level of ERK1/2 was corrected to ERK1/2 expression levels and subsequently normalized to GAPDH expression levels. The relative ERK1 and ERK2 is normalized to that of WT untreated cells obtained from three independent experiments. (B) WT and Pld1⁻/⁻ cortical in culture for 3 DIV were treated for 15 minutes with 100 ng/mL of BDNF and lysed. 35 μg of proteins was resolved by SDS-PAGE and probed with anti-phospho-mTor (Ser-2481), anti-phospho-S6K (Thr-389), anti-phospho-CREB (Ser-133) and anti-GAPDH antibodies. The levels of phospho-proteins was normalized to GAPDH expression levels and normalized to that of WT untreated cells. For pS6K quantification, lower bands levels corresponding to P70S6K were analysed. Data were averaged from three independent experiments.

tested whether PLD1 is involved in this part of the ERK-CREB pathway. Although in WT and Pld1⁻/⁻ neurons, nuclear-p-ERK1/2 staining after BDNF treatment was only modestly and non-significantly reduced (data not shown), the overexpression of PLD1-GFP nearly tripled the nuclear p-ERK1/2 signal
in both control and BDNF-treated neurons (Fig. 3A). Of note, we also observed that BDNF treatment strongly increased the intensity of nuclear p-ERK1/2 signal, an effect that was potentiated in cells over-expressing PLD1-GFP (Fig. 3A). Similarly, the overexpression of PLD1-GFP approximately doubled the nuclear p-CREB signal in control and BDNF treated cells (Fig. 3B). Interestingly the level of p-CREB signal in BDNF treated neurons was reduced by nearly 50% in the absence of PLD1 (Fig. 3C). All together these results suggest that PLD1 may contribute to the signalling pathway that leads to increased levels of p-ERK1/2 and p-CREB in the nucleus of cortical neurons after BDNF stimulation.

**PLD1, p-ERK1/2 and RSK2 on partially associated with vesicular structures.** Having shown that RSK2 regulates PLD activation in response to BDNF treatment and that PLD1 contributes to the activation of the ERK1/2-CREB signalling pathway the localization of PLD1, RSK2, and phospho-ERK1/2 was examined. However due to the lack of specific PLD1 or RSK2 antibodies capable to detect endogenous proteins for immunofluorescence experiments we overexpressed tagged versions of the proteins. After 15 minutes of BDNF treatment, 67.5% ± 2.3% of the GFP-PLD1 signal colocalized with phospho-ERK1/2 mainly in the perinuclear area, but also in dendrites and axons (Fig. 4A). Analysis of the fluorescence in the cellular extensions indicated that both staining were associated on punctuate structures (Fig. 4A). BDNF treatment of neurons overexpressing HA-RSK2 and GFP-PLD1 revealed that both proteins partially colocalized with p-ERK1/2 on vesicular structures (Fig. 4B). In subcellular fractions of BDNF-treated obtained using an OptiPrep gradient, endogenous PLD1 and RSK2 were mostly enriched at the 5-10% interphase, along with the BDNF receptor TrkB and the early endosomal markers, APP1 and Rab5 (Fig. 5A). Significant amounts of pERK1/2 were also present in this fraction (Fig. 5A), in agreement with previous findings.

Interestingly the phosphoprotein PEA15 was highly enriched in the upper gradient endosomal fraction purified from BDNF treated neurons (Fig. 5A). PEA15, predominantly expressed in the central nervous system has been recently shown to have binding sites for PLD1, RSK2 and ERK1/2, suggesting that it could be an ideal hub to integrate these signalling molecules. In line with the notion that these signalling molecules may transport the information towards the nucleus through the endocytic pathway, we also found that overexpressed GFP-PLD1 partially colocalized with pERK1/2 on late endosomal Rab7-positive vesicles after BDNF treatment (Fig. 5B).

**PEA15 is required for the BDNF-induced increase in the nuclear level of p-ERK1/2 and p-CREB.** Using an immunoprecipitation strategy, endogenous PLD1, RSK2 and ERK1/2 were coprecipitated with endogenous PEA15, in agreement with the idea that these proteins form a large complex in cortical neurons in response to BDNF treatment (Fig. 5C). To probe the notion that PEA15 may contribute to the signalling pathway that triggers elevated phospho-ERK and phospho-CREB levels in the nucleus, the endogenous level of PEA15 was reduced with small interference RNA. Hence, PEA15 levels were reduced by 73 ± 8% with siRNA#1 and by 86 ± 5% with siRNA#2 (Fig. 6A). Although reduced PEA15 expression had no effect on the nuclear levels of p-ERK1/2 and p-CREB in untreated neurons, it almost completely abolished the enhanced nuclear levels of these proteins in the response to BDNF (Fig. 6B,C). Altogether these results suggest that PEA15 is necessary to convey the PLD1-RSK2-ERK signal towards the nucleus of neurons.

**Discussion**

Neurotrophins, including BDNF are key regulators of synaptic function and neuronal development. Neurotrophins signal by binding and activating receptors with intrinsic tyrosine kinase activity. It is now well accepted that BDNF promotes neuronal development and synaptic plasticity through multiple signalling and protein synthesis-dependent mechanisms that differ between brain regions, cell types, and probably as a function of the type of induced-LTP. BDNF is crucially involved in nearly all developmental stages of neuronal circuitry including, i) survival of stem cells and progenitors, ii) neurogenesis and neuronal differentiation, iii) neuronal polarization and guidance, iv) branching and survival of differentiated neurons, and v) formation and maturation of spines and synapsese. The dynamics of BDNF-TrkB signalling and its impact on downstream signalling events are not well understood. Using cortical neuron cultures, we show here that PLD1 expression is required for phosphorylation of ERK1/2, CREB, mTor, and S6K in an acute response to BDNF. This result is in agreement with the well-known involvement of the ERK1/2-RSK2-CREB and mTor-p70/S6K pathways as major regulators of neuronal morphology and plasticity, but also with the signalling pathways downstream of PLD, although in different cell type and with different agonist. In line with a critical role of PLD1 during neuronal development, PLD1 expression was found to dramatically increase in cortical neurons after 3 days in culture, as did RSK2 expression level. BDNF treatment induced a rapid (within minutes) and transient activation of PLD activity in cultured neurons that was not seen in Rsk2−/− neurons, arguing that RSK2 acts upstream of PLD1. Having previously shown that RSK2 can phosphorylate PLD1 on Thr147 during neurosecretion, one can speculate that RSK2 phosphorylation of PLD1 is also required for the activation of PLD in cortical neurons treated with BDNF. Of note, in lyastes of cortical neurons cultured for 10 and 12 DIV a second higher molecular weight band positive for PLD1 that may correspond to a phosphorylated form of PLD1 was detected. This could, however, not be tested experimentally because the anti-PLD1-phospho-Thr147 antibody does not recognize the mouse phospho-PLD1 (data not shown).
Figure 3. PLD1 regulates the nuclear level of p-ERK1/2 and p-CREB. 2 DIV WT cortical neurons were transfected with PLD1-GFP and after 24 hours treated for 15 minutes with 100 ng/mL of BDNF. After fixation cells were stained with anti-phospho-ERK1/2 (A) or anti-phospho-CREB (B) antibodies and the nucleus stained with DAPI (not shown). (C) WT and Pld1−/− cortical neurons in culture for 3 DIV were treated for 15 minutes with 100 ng/mL of BDNF. After fixation cells were stained with anti-phospho-CREB and anti-ß-tubulin antibodies, and the nucleus was stained with DAPI (not shown). The level of nuclear phospho-ERK1/2 (A) or phospho-CREB (B,C) fluorescence intensity was normalized to the nuclear area revealed by DAPI staining from at least 30 cells per condition. Bar = 25 μm.
In line with this possibility, PLD1 was recently found in a screen of phosphorylated proteins in mouse synaptosomes.

The BDNF-activated intracellular signal transduction pathways probably mediate neuronal adaptation, in part by modifying existing proteins through phosphorylation for instance, but also by activating transcription factors that regulate gene expression. However, the signal transduction pathways that connect signals generated at synapses with transcriptional responses in the nucleus are not well understood. In the present report, BDNF treatment increased the nuclear accumulation of phospho-ERK1/2 and phospho-CREB in a PLD1-dependent manner. Furthermore, PLD1, RSK2, and ERK1/2 were found colocalized on vesicular structures present both in neuronal axons and dendrites. These structures were positive for Rab5, Rab7, and APP1, indicating that they are part of the recycling endosomal system. A model for the regulation of ERK1/2 phosphorylation by cell surface receptors involves Ras–Raf complexes bound to the surface of endosomes, where scaffolding complexes involving Ras, cRaf-1, MEK and ERK1/2 are formed. Complete activation and coupling of this cascade requires endocytosis, a process that is also modulated by phosphatidic acid (PA) produced by PLD. Therefore, the current data suggest that RSK2 and PLD1 may also be part of this complex on recycling endosomes.

In line with the finding that the scaffolding protein PEA15 contains PLD1, RSK2, and ERK1/2 binding sites, the present immunoprecipitation results validate the notion that BDNF triggers the formation of a multiprotein complex containing these four partners in cortical neurons. Furthermore, PEA15 silencing...
Figure 5. PLD1, p-ERK1/2 and PEA15 are found on late endosomal vesicles. (A) 3 DIV neurons treated for 15 minutes with 100 ng/mL of BDNF were lysed and subjected to subcellular fractionation by velocity centrifugation on OptiPrep gradient (5-10-15-20-25%). Fractions were collected and after Western blotting probed with anti-TrkB, anti-PLD1, anti-APPL1, anti-RSK2, anti-pERK1/2, anti-Rab5, and anti-PEA15 antibodies. Similar observations were obtained on two independent experiments. (B) 2 DIV WT cortical neurons were transfected with PLD1-GFP and 24 hours later treated for 15 minutes with 100 ng/mL BDNF. After fixation, cells were stained with anti-phospho-ERK1/2 and anti-Rab7 antibodies. Arrows indicate vesicular structures positives for PLD1, Rab7, and phospho-ERK1/2. Bar = 25 μm. (C) 3 DIV neuronal cultures untreated (−) or treated (+) for 15 minutes with 100 ng/mL BDNF were lysed, and PEA15 was immunoprecipitated. Pre-immunoprecipitation, immunoprecipitated and post-immunoprecipitated samples were probed with anti-PEA15, anti-PLD1, anti-RSK2, anti-ERK1/2, and anti-GAPDH antibodies. GAPDH was used a negative control of immunoprecipitation. Note that the amount of PEA15 immunoprecipitated after BDNF treatment varied and that the amount of ERK1/2 coimmunoprecipitated varied accordingly. Similar observations were replicated twice.
significantly affected the levels of nuclear phospho-ERK1/2 and phospho-CREB, providing evidence that this quadripartite complex is probably participates in the retrograde transport of the molecular modules involved in BDNF signalling.

Alterations in BDNF levels are associated with neurodegenerative disorders (including Alzheimer's disease, Huntington's disease and epilepsy), neuropsychiatric disorders (including depression, anxiety disorders, bipolar disorders, schizophrenia and addiction) and obesity. The hallmark of BDNF deficiency is synaptic degeneration, whereas increasing BDNF levels promote synaptic repair in preclinical models. Moreover, BDNF could potentially be used to treat diseases in which alterations in its levels are not directly involved in the pathogenesis (for instance, in Parkinson's disease, amyotrophic lateral sclerosis, stroke and spinal cord injury). BDNF is a highly charged protein that does not readily cross the blood–brain barrier making CNS delivery a challenge. So targeting molecules involved in BDNF signalling may represent alternative strategies. The current data indicating a role for PLD1 in BDNF signalling opens up the possibility to compensate decreased or increased PLD1 signalling/activity in this pathophysiological context. This is especially appealing since several reports have implicated PLD in neurodegenerative disorders (reviewed in 43).

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Author Contributions
M.R.A. designed, performed experiments and analysed results. T.T. performed experiments. A.H., D.S. and B.N. provided useful reagents. N.V. designed and analysed the experiments and wrote the manuscript. All authors reviewed the manuscript.

Additional Information
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