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The lipase activity of phospholipase D2 is responsible for nigral neurodegeneration in a rat model of Parkinson's disease

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Abstract

Phospholipase D2 (PLD2), an enzyme involved in vesicle trafficking and membrane signaling, interacts with α-synuclein, a protein known to contribute in the development of Parkinson disease. We previously reported that PLD2 overexpression in rat substantia nigra pars compacta (SNc) causes a rapid neurodegeneration of dopamine neurons, and that α-synuclein suppresses PLD2-induced nigral degeneration (Gorbatyuk et al., 2010). Here, we report that PLD2 toxicity is due to its lipase activity. Overexpression of a catalytically inactive mutant (K758R) of PLD2 prevents the loss of dopaminergic neurons in the SNc and does not show signs of toxicity after 10 weeks of overexpression. Further, mutant K758R does not affect dopamine levels in the striatum. In contrast, mutants that prevent PLD2 interaction with dynamin or growth factor receptor bound protein 2 (Grb2) but retained lipase activity, continued to show rapid neurodegeneration. These findings suggest that neither the interaction of PLD2 with dynamin, which has a role in vesicle trafficking, nor the PLD2 interaction with Grb2, which has multiple roles in cell cycle control, chemotaxis and activation of tyrosine kinase complexes, are the primary cause of neurodegeneration. Instead, the synthesis of phosphatidic acid (the product of PLD2), which is a second messenger in multiple cellular pathways, appears to be the key to PLD2 induced neurodegeneration. The fact that α-synuclein is a regulator of PLD2 activity suggests that regulation of PLD2 activity could be important in the progression of Parkinson disease.

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Conflict of Interest

N.M. is an inventor of patents related to rAAV technology and owns equity in gene therapy companies that are commercializing AAV for gene therapy applications. The remaining authors declare that they have no competing financial interest.

Keywords

PLD2; alpha-synuclein; Parkinson disease; AAV transgenics; phosphatidylcholine; neurodegeneration

Introduction

Phospholipase D (PLD) is a family of ubiquitous enzymes that convert phosphatidylcholine (PC) into phosphatidic acid (PA) and soluble choline. Two mammalian phospholipase D genes have been described so far, PLD1 and PLD2 (Hammond et al., 1995; Colley et al., 1997). PLD2 has been implicated in Parkinson Disease (PD), a neurodegenerative brain disorder characterized by a loss of dopamine-secreting neurons in the substantia pars compacta (SNc) and depletion of dopamine (DA) in the striatum (Davie, 2008). We have shown previously that AAV-mediated overexpression of PLD2 in the rat SNc leads to the loss of DA neurons, similar to that seen in PD (Gorbatyuk et al., 2010). It has also been found that PLD2 interacts with α-synuclein (α-syn) (Jenco et al., 1998), a small protein implicated in the pathogenesis of Parkinson disease. Interaction with α-syn has been demonstrated both *in vitro* and *in vivo* (Duda et al., 2000; Gorbatyuk et al., 2010). *In vitro*, α-syn acts as a potent inhibitor of PLD2 phospholipase activity and *in vivo*, α-syn inhibits PLD2 neurotoxicity when both proteins are expressed in dopaminergic neurons of the SNc (Gorbatyuk et al., 2010). However, the exact relation of PLD2 with PD as well as its physiological role in nigral neurons is still unknown. In mammals, PLD2 has been proposed to play a role in endocytosis, exocytosis, vesicular trafficking, cytoskeletal reorganization and signal transduction via different interaction domains of the protein or via PA, the product of PLD2 (Cockcroft, 2001; Jenkins and Frohman, 2005).

The amino acid sequence of PLD2 can be divided in two parts: a segment containing the motifs necessary for lipase catalysis and a segment containing the regulatory sequences that interact with other cellular proteins, including α-syn (Fig. 1). The catalytic active site, located in the C terminal sequence of PLD2, has four conserved regions, where regions II and IV contain the HDK motifs that are responsible for enzymatic activity (Hammond et al., 1995; Morris et al., 1996). This region also has a phosphatidylinositol 4,5-bisphosphate (PIP2) binding site, an essential cofactor required for lipase activity (Brown et al., 1993). As noted earlier, the PLD2 catalytic site generates PA, which can act in different ways. It can recruit proteins like Raf or mTOR that are activated as a result of the recruitment to mediate pathways such cell proliferation and cell survival (Rizzo et al., 1999; Fang et al., 2001; Zhao et al., 2007). It can also be converted to other phospholipids such as lysophosphatidic acid (LPA) or diacylglycerol (DAG), which are involved in a wide range of biological processes, including cell growth, differentiation, migration, or inflammatory processes (Jenkins and Frohman, 2005; Cazzolli et al., 2006). In addition, PA is a negatively charged phospholipid with a small head group that promotes negative membrane curvature. This property is thought to facilitate membrane vesicle fusion and fission (Kooijman et al., 2003; Huang et al., 2005), which is believed to be a key step in vesicle formation in the Golgi, in endocytosis of receptors and in exocytosis (Jones et al., 1999; Jenkins and Frohman, 2005).

The second segment, located in the N-terminus of the PLD2 sequence, controls the regulation of the lipase activity and the location of the protein (Jenkins and Frohman, 2005). This sequence has binding sites for proteins, such as α-syn (Ahn et al., 2002), phospholipase C (PLC) (Jang et al., 2003), EGFR (Slaaby et al., 1998), Grb2 (Di Fulvio et al., 2007) and dynamin (Lee et al., 2006), as well as for phospholipids (Sugars et al., 2002).

The interaction of PLD2 with Grb2 and dynamin have been well characterized. Grb2 is an adaptor protein involved in signal transduction of tyrosine kinase receptors, including EGFR (Di Fulvio et al., 2007) and dopamine D3 receptors (Oldenhof et al., 2001). Grb2 is a critical signal transducer of EGFR, linking EGFR to the Sos/Ras/Erk pathway (Di Fulvio et al., 2007; Zhao et al., 2007) as well as PLD2.

Dynamin, is a GTPase involved in vesicle formation that catalyzes the pinching off of vesicles (Hinshaw, 2000). PLD2 stimulates dynamin GTPase activity (Lee et al., 2006), which promotes receptor-mediated endocytosis (Schmid et al., 1998), internalization of caveolae (Henley et al., 1998) and synaptic vesicle recycling (Hannah et al., 1999).

As mentioned earlier, PLD2 overexpression in DA neurons of the SNc has been found to cause nigral-striatal degeneration. Several mutations have been reported that disrupt interaction of PLD2 with other cellular proteins or abolish catalytic activity. Substitution of arginine for lysine at amino acid 758 (K758R) in the C terminal HKD motif results in loss of phospholipase activity. This mutant is unable to produce PA but the protein is fully stable (Sung et al., 1997). Similarly, mutation of PLD2 residue Y179 decreases the binding between PLD2 and Grb2, affecting the signaling of tyrosine kinase receptors (Di Fulvio et al., 2006). In addition, mutation of the next amino acid R180K prevents the interaction of PLD2 with dynamin, blocking the role of PLD2 during formation/fusion of membrane vesicles (Lee et al., 2006). In order to determine which of these functions of the PLD2 protein causes toxicity in rat SNc, we made mutations in PLD2 sequences responsible for phospholipase activity (K758R), for Grb2 interaction (Y179F) or for dynamin interaction (R180K), and studied their effects on toxicity in rat SNc. We found that overexpression of the mutants Y179F and R180K caused neurodegeneration of nigral dopaminergic neurons that was identical to that seen with wild type PLD2. However, no sign of toxicity was seen with K758R, the lipase active site mutant. Our findings support the idea that the phospholipase activity of PLD2 is responsible for the neural toxicity of PLD2 in our model of vector-mediated neurodegeneration in the rat SNc.

Experimental procedures

DNA constructs

The plasmid containing wild type PLD2 open reading frame (ORF) with an N-terminal HA tag in frame, was made previously as described in (Gorbatyuk et al., 2010). Three mutants of PLD2 were made: Y179F, R180K and K758R (Fig. 1). The plasmids containing the mutations were generated by PCR mutagenesis using the QuickChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Briefly, after PCR amplification, products were digested with DpnI, transformed into SURE cells (Agilent Technologies, Santa Clara, CA), plated on ampicillin-containing agar plates and grown overnight at 37ºC.

Single colonies were picked and grown up overnight in liquid LB with ampicillin (100 μg/mL). Following DNA purification, the plasmids were sequenced and used for virus preparation. The mutations Y179F, R180K and K758R were verified by sequencing the full open reading frame of the PLD2 sequence. The plasmid containing the GFP ORF, called pTRUF11, was previously described (Burger et al., 2004).

Virus preparation

Recombinant Adeno-associated virus (rAAV) vectors were provided by the Powell Gene Therapy Center Core at the University of Florida. rAAV were produced as previously described (Zolotukhin et al., 2002; Burger et al., 2004). Briefly, plasmids containing PLD2, Y179F, R180K, K758R or GFP were co-transfected with pKrap5-5A (a plasmid that contains the AAV5 rep and the AAV5 capsid genes (Chiorini et al., 1999) and the adenovirus E4, VA, and E2a helper regions required for AAV production (Xiao et al., 1998)). The transfections were done in HEK293 cells. The recombinant virus was purified by iodixanol step gradient followed by monoQ column purification. The vectors were subsequently concentrated and buffer exchanged with lactated Ringer's using Apollo 150 kDa concentrators (Orbital biosciences, MA, USA. Cat. AP2015010) and the virus was stored at −80ºC. Virus titers (vector genomes (vg) per mL were determined by dot blot assay (Zolotukhin et al., 2002). For every experiment, the titer of all the viruses was adjusted into the range between 1.17×10^{12} and 2.92×10^{12} vg/mL.

Cell cultures

HEK293 cells were maintained at 37°C and 5% CO2 in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Animals

The animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida. Sprague Dawley rat of 7–8 weeks of age were used for the experiments. 20 rats were used to measure PLD activity in SNc, 26 animals for the 4-week stereological estimation of the total TH-positive neurons in the SNc and for DA, DOPAC and HVA measurements in the striatum. 16 animals were used for TH immunohistochemistry of the striatum. 26 animals were used for the 10-week stereological estimation of TH neurons in SNc and TH expression in striatum by western blot analysis.

Intracerebral injections

All surgical procedures were performed using aseptic techniques and isoflurane gas anesthesia. Anesthetized rats were placed in the stereotaxic frame (Kopf Instruments, Tujunga, CA, USA), and 3 μL of virus solution injected into the right SNc (5.3 mm posterior from bregma, 2.4 to midline and 6.7 ventral to dura) through a pulled glass micropipette with an inner diameter ~30–40 μm at a rate of 0.5 μL/minute. The needle was left in place for 5 min prior to withdrawing from the brain. Injections were done only in the right side of

the brain; the left side was used as the uninjected control. Animals were sacrificed at 2, 4 or 10 weeks after injection.

Tissue preparation

Animals were deeply anesthetized with pentobarbital (Beuthanasia-D) injection. Brains were removed and divided in two parts by a coronal blade cut at approximately 3.5 mm posterior to bregma.

For PLD activity assays—The caudal part containing the SNc was frozen on dry ice and kept at −80ºC. Coronal sections (150 μm thick) were cut on a freezing stage sliding microtome. From each brain slice, SNc from each hemisphere was dissected out into two separate tubes, frozen on dry ice and kept at −80ºC until assayed for PLD activity.

Dopamine measurements and immunoblotting—The rostral part of the brain was used immediately to dissect the striatum from each hemisphere and saved in two separate tubes. Each hemisphere was homogenized and separated into two additional tubes, one for protein expression and the other one for Dopamine (DA), 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) content. The tissue pieces were weighed, frozen on dry ice and kept at −80ºC until assayed for immunoblotting or DA measurement.

For histology—Both parts were fixed in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4 overnight. Forty-micrometer-thick brain coronal were cut on a vibratome (Leica Microsystems) and processed for immunohistochemistry.

PLD lipase activity *in vitro*

Each mutant was transfected into HEK293. 24 hours later, cell were collected and frozen until processing. For the analysis, cells were resuspended in “reaction buffer” provided in the Amplex Red phospholipase assay kit (A12219, Molecular Probes) followed by three freeze/thaw cycles. Subsequently, cell extracts were used to quantify PLD2 activity following manufacturer’s protocol.

PLD lipase activity in tissue

Frozen SNc tissue was homogenized in ice cold buffer containing 50 mM Tris-HCl, 5 mM CaCl, pH 8.0. 30 μg of protein from the homogenized samples were used to determine PLD activity with the Amplex Red Phospholipase D Assay Kit (A12219, Molecular Probes) according to the manufacturer’s protocol.

DA, DOPAC and HVA measurements

Samples were processed and the content of dopamine (DA), 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured by the Neurochemistry Core Lab., Vanderbilt University Medical Center, Nashville, TN, as previously described (Crabtree et al., 2013).

Immunoblotting

Tissue were suspended in 100–300 μL of lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.2 mM EDTA) containing a protease inhibitor cocktail (04693159001, Roche) and homogenized for 10 seconds. Each sample was adjusted to a final concentration of 1% NP-40, 0.1% SDS, incubated on ice for 30 min, and centrifuged for 15 min 10000 g at 4ºC to clear the debris. Protein concentrations were determined by Bradford protein assay. Fifty micrograms of each lysate were loaded into Bio-Rad precast 4–20% Criterion TGX gels (Bio-Rad, 567-1094), transferred to LFP PVDF (GE Healthcare, RPN1416LFP) or nitrocellulose (GE Healthcare, 10401197) membranes, and immunoblotted. Membranes were blocked for 1 hour with 5% Blotting-Grade Blocker (Bio-Rad, 170-6404) in PB - 0.5% Tween 20 (Fisher Scientific, BP337-500) following by 4ºC overnight incubation with primary antibodies: mouse anti-TH (1:4000, MAB318, Millipore), rabbit anti-TH (1:4000, AB152, Millipore), mouse anti-GAPDH (1:2000, AB9484, Abcam). Membranes were washed three times for 5 min each in 1 PBS- 0.5% tween and incubated with secondary antibodies for 1 hour at room temperature: Goat Cy5-anti-mouse IgG (1:2000, PA45009, GE Healthcare), Goat Cy5-anti-rabbit IgG (1:4000, PA45011, GE Healthcare) or sheep horseradish peroxidase(HRP)-anti-mouse IgG HRP (1:5000, NA931, GE Healthcare). After three 5-min washes, membranes were analyzed by fluorescence with a Typhoon 9200 Imager (Amersham) or by chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500, Millipore).

Immunohistochemistry

Immunostaining was carried out on free-floating sections as follows: Endogenous peroxidase activity was removed by incubation in 0.5% H2O2 and 10% methanol for 15 min. at RT. Sections were incubated 0.1% Triton-X100, 3% normal horse serum (NHS) in PBS for 1 hour at RT followed by incubation with one of two primary antibodies, mouse anti-TH (1:4000, MAB318, Millipore) or mouse anti-HA-tag (1:300, Cat. 2367, Cell Signaling) diluted in 0.1% Triton X and 1% NHS in PBS for 16 hours at 4ºC. Sections were washed three times for 10 min each in PBS and incubated with horse Biotinylated-anti-mouse (1:500, BA-2001, Vector Laboratories) diluted in 0.1% Triton X and 1% NHS in PBS for 2 hours at room temperature. Sections were stained using NovaRED Peroxidase (HRP) Substrate Kit (Sk-4800). After washing, sections were mounted on slides and dehydrated in ascending concentrations of ethanol, cleared with xylene and coverslipped with Permount (S70104, Fisher Scientific).

Unbiased Stereology

The unbiased stereological estimation of the total number of the TH+ neurons in SNc was performed by the optical fractionator method, as previously described (West et al., 1991; Kirik et al., 1998), with the MicroBrightfield Stereo Investigator System. The estimate of the total number of neurons and coefficient of error due to the estimation was calculated according to the optical fractionator formula as described in (West et al., 1991).

Statistical Analysis

GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. One single data point per animal was used in every statistical analysis across the study. Data were analyzed using one-way analysis of variance (ANOVA) as parametric test followed by Tukey test to assess statistical significances, or Kruskal-Wallis non-parametric test followed by Dunn's test as multiple comparisons test.

Shapiro-Wilk test was employed to examine for normal distributions and Brown-Forsythe for equal variances. Statistical significance was defined when $P < 0.05$. All data are presented as mean ± standard error of mean (SEM).

Results

K758R mutation blocks PLD2 lipase activity

Previous reports have shown that a mutation at amino acid position 758 eliminates PLD2 lipase activity *in vitro* but not the mutations at amino acid 179 and 180 (Sung et al., 1997; Park et al., 2004; Di Fulvio et al., 2006). To confirm these results, we examined the PLD activity of our constructs in culture and rat brain. HEK293 cells were transfected with plasmids containing wt PLD2, Y179F, R180K, K758R or GFP and at 24 hours post transfection, cells were collected and assayed for PLD activity (Fig. 2A). Whereas wt PLD2, Y179F and R180K groups showed elevated PLD activity, cells transfected with K758R exhibited similar levels of PLD activity as GFP control. For the *in vivo* test, AAV viral vectors expressing wt PLD2, Y179F, R180K, K758R or GFP were injected in the SNc of rats. Only the right hemisphere of the brain was injected and the other side was kept as uninjected control. Two weeks after the injection, all nigral tissues were collected, lysed and assayed for PLD activity (Fig. 2B). For each animal, the level of PLD activity in the injected side was compared with the contralateral uninjected side. The results showed that tissues expressing wt PLD2, Y179F and R180K exhibited significantly higher levels of PLD activity (112.1% ± 2.2, 117.7% ± 8.9 and 117.6% ± 6.6, respectively) on the injected side vs the uninjected side, indicating that these constructs increase total PLD activity in the SNc. In contrast, there was no significant change in PLD activity in K758R and GFP injected animals (89% and 94%, respectively), showing that mutant K758R does not contribute to the total PLD activity in the rat SNc.

Overexpression of K758R mutant in the SNc does not cause neurodegeneration

To see if any of the PLD2 mutants lost the ability to promote neurodegeneration in the SNc, AAV viruses expressing HA tagged wt PLD2, Y179F, R180K or K758R were injected unilaterally in SNc of rats. GFP expressing virus was used as a control. At 4 weeks after injection, immunohistochemistry with anti-HA antibody was employed to localize the expression of wt PLD2 and the mutants. The HA epitope was inserted at the N-terminus of PLD2 to distinguish exogenous PLD2 and mutants from endogenously expressed PLD2. As expected, expression of exogenous PLD2 was located in the SNc only on the injected side (Fig. 3F). The same location was found for the mutants Y179F, R180K and K758R (Fig. 3G, H and I, respectively), as well as GFP (Fig. 3J). Virtually all of the SNc showed HA expression (GFP expression in the control), as well as the immediate surrounding area,

including part of the substantia nigra pars reticulata (SNr) and ventral tegmental area (Vta). It has been shown previously (Burger et al., 2004; Paterna et al., 2004; McFarland et al., 2009) that the AAV5 serotype used in our experiment is able to transduce virtually all the neurons of the rat SNc, that expression occurs predominantly in neurons rather than astrocytes, and that it produces little if any inflammatory response or gliosis (McFarland et al., 2009; Ulusoy et al., 2009). Thus, phenotypes found in this work can be considered the result of genetic changes in neurons of the SNc rather than nonspecific effects caused by AAV injection or transduction of glial cells.

Immunohistochemistry with a anti-TH antibody revealed a loss of TH-positive cells in animals injected with wt PLD2 (Fig. 3A, arrows), which was similar to what we reported previously (Gorbatyuk et al., 2010). The same phenotype was found in animals injected with the mutants Y179F (Fig. 3B, arrows) and R180K (Fig. 3C, arrows). In contrast, animals that had been injected with the K758R mutant (Fig. 3D), which destroys the phospholipase active site, showed essentially no loss of TH positive cells when compared to the uninjected side. Unbiased estimation of nigral TH positive cells at 4-week post-injection revealed that animals injected with wt PLD2, Y179F and R180K retained 40% ± 3, 38% ± 10 and 47% ± 10 of TH^+ cells respectively (Fig. 3K), while AAV-mediated expression of either the catalytic site mutant, K758R, or the control GFP construct did not lead to neuronal loss (96% ± 4 and 98% ± 7 remaining TH positive cells, respectively).

These results suggested that interaction of PLD2 with Grb2, and presumably the kinases that use Grb2 as an essential scaffold protein, is not essential for PLD2 induced neurotoxicity in the SNc. Similarly, the interaction of PLD2 with dynamin, a key factor in endosome formation and DA uptake, is also not involved in PLD2 induced acute toxicity in SNc. In contrast, the enzymatic activity of PLD2 or one of its downstream effects are due to phosphatidic acid synthesis or the increase in membrane fluidity seemed to be responsible for neurodegeneration.

K758R mutant does not show an effect in the striatum

Even though there was an apparent lack of neurodegeneration of K758R mutant in the SNc, we wondered if projections of the nigral TH^+ neurons can be affected in the striatum. To test this hypothesis, sections of striatum were immunostained against TH. We found reduction of TH staining on the injected side of wt PLD2 animals (Fig. 4A), as well as animals injected with Y179F (Fig. 4B) and R180K (Fig. 4D) expression constructs. However, there was no obvious difference in TH staining between injected and uninjected sides of animals overexpressing mutant K758R (Fig. 4E) or GFP (Fig. 4C). Thus, K758R mutant doesn't alter TH protein levels in the striatum.

Effect of mutant gene expression on striatal DA levels

We next hypothesized that even though TH expression in striatum was not altered, DA levels or its metabolites 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) could be affected by the presence of mutant K758R. DA, DOPAC and HVA were extracted from striatal tissue, fractionated by High Performance Liquid Chromatography (HPLC), and measured by electrochemical detection. We found that levels of DA correlate to levels of TH

production (Fig. 4F). wt PLD2, Y179F and R180K injected animals showed severe reduction (about 50–60%) of DA, DOPAC and HVA levels on the injected side. In contrast, no significant difference in levels of DA, DOPAC or HVA was found between injected and uninjected sides of animals expressing K758R or GFP.

Longer expression of K758R mutant does not affect SNc or striatum

As previously reported, overexpression of other proteins, such as human α-synuclein, produced a slow onset of neurodegeneration that became significant after time periods much longer than 4 weeks (Gorbatyuk et al., 2008). To determine if this could be the case with the PLD2 active site mutant, we examined toxicity 10 weeks postinjection. (Fig. 5). Immunohistochemistry with anti-HA antibody revealed that expression of wt PLD2 (Fig. 5F) as well as the mutants (Fig 5G–I), and GFP (Fig. 5J) was still robust at 10 weeks. TH-immunoperoxidase staining revealed that while wt PLD2, Y179F and R180K animals (Fig. 5A, B, and C, respectively) showed marked neurodegeneration of TH+ neurons in the SNc (arrows), there was no significant neurodegeneration in the K758R group (Fig. 5D). Unbiased estimation of TH-positive cells in animals injected with wt PLD2, Y179F and R180K showed a 40–50% loss of TH^+ neurons in the SNc (38% ± 12, 44% ± 9, 49% ± 8 neurons remaining, respectively) (Fig. 5K). Again, no significant neurodegeneration was found in animals injected with K758R mutant vectors (109% ± 13). A non-significant loss of TH neurons was also found in the GFP control group at 10-week postinjection (77% ± 12), confirming earlier reports that GFP was moderately toxic when overexpressed in the brain (Klein et al., 2006; Yang et al., 2016). We concluded that the K758R mutant without lipase activity does not cause neurodegeneration in the SNc, even at 10-week postinjection.

To analyze if TH positive synaptic projections to the striatum were affected, pooled striatal tissues obtained at 10 weeks were also analyzed by quantitative immunoblotting with anti-TH antibody (Fig. 6). Levels of TH protein were between 53% and 65% in the wt PLD2, Y179F and R180K groups (53.5% ± 3.9, 65.4% ± 5.5, 59.5% ± 5.0, respectively). No reduction of TH levels was found in K758R group or GFP control group (98.0% ± 3.8, 99.9% ± 2.7, respectively), consistent with the unbiased count of TH positive neurons found in SNc.

Discussion

We confirmed our previous finding that overexpression of PLD2 induces a rapid loss of TH-positive cells in the SNc, with a loss of >50% of DA neurons in the first 4 weeks after viral vector injection (Fig. 3) (Gorbatyuk et al., 2010). PLD2 converts phosphatidylcholine to choline and phosphatidic acid (PA), in the presence of PIP2, an essential cofactor. PLD2 is involved in a variety of signal transduction pathways either by directly interacting with other cellular proteins or by the synthesis of PA, which acts as a second messenger. In addition, conversion of phophatidylcholine to PA is believed to make the membrane more fluid and to promote membrane curvature, thereby, promoting vesicle formation or vesicle fusion. To learn more about the mechanism driving PLD2 mediated neurodegeneration, we analyzed three classes of PLD2 mutants.

One of these, mutant Y179F, has been reported to produce a 70% reduction in interaction between PLD2 and Grb2 (Di Fulvio et al., 2006). The Grb2 - PLD2 interaction is involved in a variety of signaling pathways, including receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR) and G protein-coupled receptors, such as the metabotropic glutamate receptor (mGlu) (Bhattacharya et al., 2004; Dhami and Ferguson, 2006; Di Fulvio et al., 2007; Zhao et al., 2007). Grb2 serves the function of an adaptor protein which binds multiple proteins involved in signal transduction via its SH2 and SH3 domains, thereby bringing them in close contact. The PLD2 Y179 residue is one of two amino acids that interacts with SH2 domains of Gbr2. Grb2 in turn binds to son of sevenless (SOS), a guanine nucleotide exchange factor (GEF) that activates the ras/erk/mapk signaling pathway. Grb2 also binds to other signaling proteins, for example WASp (Wiscott-Aldrich syndrome protein), which activates Arp2/3. The heterotrimer PLD2-Grb2-WASp then enables actin nucleation and phagocytosis. The fact that expression of mutant Y179F shows the same levels of toxicity as wild type PLD2 suggests that the PLD2-Grb2 interaction is not the main cause of nigrostriatal degeneration and essentially rules out a number of potential signaling pathways. However, Grb2 has also been shown to bind to PA, the product of the PLD2 lipase activity, and this has been shown to be an alternative mechanism by which PLD2 and Grb2 can assemble tyrosine kinase receptor driven signaling complexes at the membrane. Thus, the fact that reduction of the direct Grb2-PLD2 interaction does not reduce neurodegeneration does not rule out an indirect role for Grb2 and its signaling partners.

Another set of well characterized mutations are R128 and R197 in PLD1 or R110/R180 in PLD2. These conserved arginines act as arginine fingers that stabilize the GTP hydrolysis transition intermediate in GTP activating proteins (GAPs). PLD2 is believed to be a non-conventional GAP that activates GTP hydrolysis by dynamin during the process of vesicle formation. Mutation of either arginine leads to a 50% reduction of dynamin GTPase activity *in vitro* and an almost complete inhibition of epidermal growth factor receptor (EGFR) endocytosis when cells were treated with epidermal growth factor (EGF) (Lee et al., 2006). The interaction of PLD2 with dynamin is consistent with PLD2's enzymatic role, the hydrolysis of the common membrane lipid phosphatidylcholine to PA. PA increases membrane fluidity and curvature, which is believed to facilitate vesicle formation. The fact that R180K, which abolishes PLD2 interaction with dynamin, has the same toxic phenotype as wt PLD2 establishes that PLD2 activation of dynamin is not involved in nigrostriatal neurodegeneration. It also suggests that any role PLD2 may have in endocytosis is not likely to be involved in neurodegeneration (however, see below).

The other well characterized mutations are the ones that block lipase activity. PLD2 has a well characterized phospholipase active site, the HKD motif ($HXKX_4DX_6GSXN$). A mutation at position K758R is unable to produce PA but the protein is fully stable (Sung et al., 1997). When this mutant was tested *in vivo*, there was no sign of toxicity after 4 weeks of expression in the SNc, in contrast to wt PLD2 and the mutants Y179F and R180K (Fig. 3). Similarly, longer times of expression (Fig. 5, 10 weeks) also showed no sign of neurodegeneration, confirming that PLD2-lipase activity is essential for neurodegeneration. The fact that the single mutation K758R completely blocks the toxicity of PLD2 suggests that PA signaling is the most probable mechanism of toxicity.

The search for the mechanism of PA toxicity is complicated because PA signaling has been implicated in several cellular processes that include vesicular trafficking, transformation, anti-apoptosis, cell growth and division, innate immune response, and cell migration. PA has been shown to bind to at least 45 proteins, many of which are downstream regulatory proteins, such as kinases, phosphatases, G-protein regulators, and phosphodiesterases (Bruntz et al., 2014). PA binding has been shown to enhance activity of its target proteins (for example, in the case of mTOR (Fang et al., 2001)), to promote translocation of the target protein (for example, Raf (Ghosh et al., 1996; Rizzo et al., 1999)) or to inhibit the target protein (for example, protein phosphatase (PP1) (Jones and Hannun, 2002). Moreover, PA is a precursor for lysophosphatidic acid (LPA) and diacylglycerol (DAG) (Oude Weernink et al., 2007), both of which are themselves signaling molecules. In recent years, PLD2 signaling has been linked to tumor cell survival and metastasis via modification of angiogenesis, cell cycling, apoptosis, autophagy or cell invasion processes (Bruntz et al., 2014). It is not immediately obvious, therefore, why an increase in PLD2 would lead to dopaminergic cell death. We note, however, that disruption of any of the pathways that control cell division, in principle, might lead to cell death if the cell's antiapoptotic signaling pathways are overwhelmed.

In summary, we have shown that overexpression of PLD2 in rat SNc leads to a severe loss of dopaminergic neurons. The loss appears to be due to the synthesis of the product of PLD2, the signaling molecule phosphatidic acid. The identification of PLD2 in a novel pathway of dopaminergic neurodegeneration suggests that regulation of PA may have a role in the onset of PD. Previous work showing that α-syn, a protein implicated in Parkinson's Disease, is a regulator of PLD2 activity and inhibits PLD2 induced toxicity is consistent with a possible role for PLD2 in PD.

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Abbreviations

PLD2
phospholipase D2

α-syn
α-synuclein

SNc
substantia nigra pars compacta

PD
Parkinson disease

TH
tyrosine hydroxylase

DA
dopamine

DOPAC
3,4-Dihydroxyphenylacetic acid

HVA
homovanillic acid

AAV
adeno-associated virus

Grb2
growth factor receptor bound protein 2

PC
phosphatidylcholine

PA
phosphatidic acid

LPA
lysophosphatidic acid

DAG
diacylglycerol

PLC
phospholipase C

epidermal growth factor receptor
EGFR

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Highlights

- PLD2 overexpression in substantia nigra causes a rapid neurodegeneration of dopamine neurons.
- PLD2 interactions with Grb2 or dynamin are not the primary cause of neurodegeneration.
- PLD2 toxicity in the Substantia nigra is due to its lipase activity.

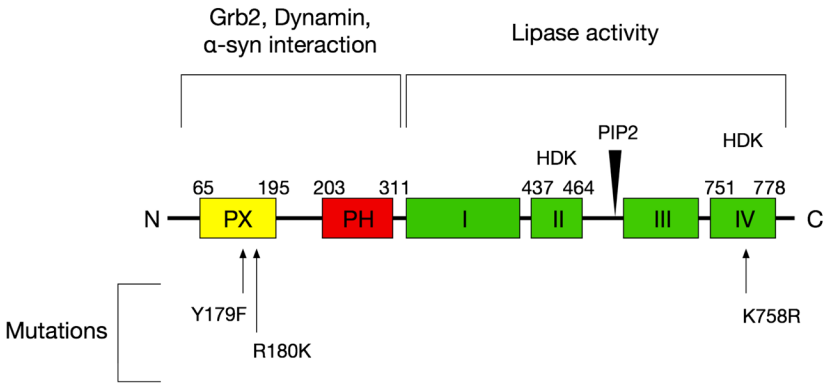

Fig. 1. A map of rat PLD2

PX and PH are plecstrin and hox domains involved in protein and lipid interactions. Mutation Y179F affects the interaction of PLD2 with Grb2. Mutation R180K alters the interaction with Dynamin. Regions I–IV are required for enzyme activity, and contain the conserved catalytic sites HKD, as well as the PIP2 binding site. Mutation K758R produces a PLD2 protein with no lipase activity.

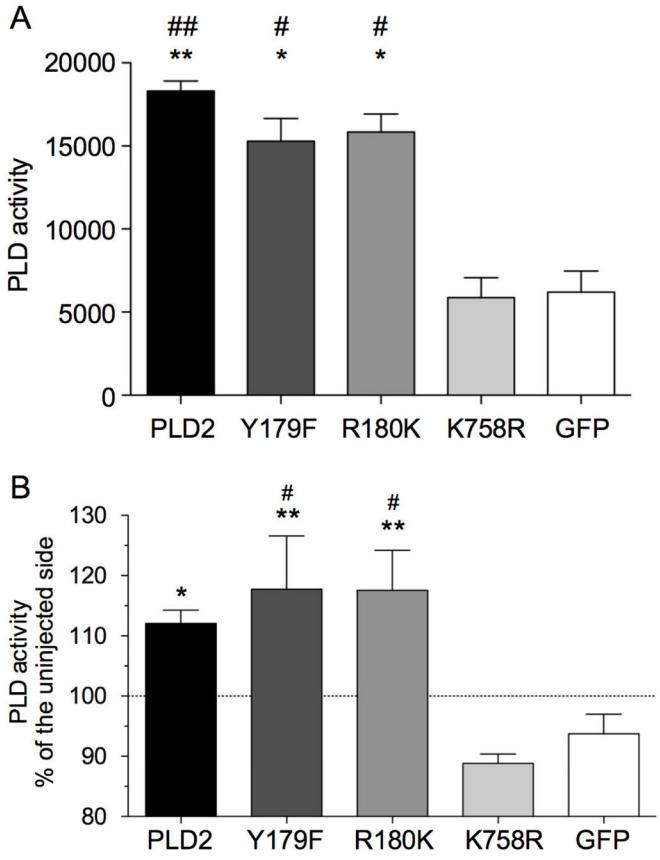

Fig. 2. Lipase activity of wt PLD2 and mutants

(**A**) HEK293 cells were transfected with the DNA constructs PLD2, Y179F, R180K, K758R or GFP. 24 hours, cells were collected, cellular extracts were processed and levels of PLD2 activity were determined by the Amplex Red PLD assay kit. Results are expressed in relative fluorescence units (RFU), as mean + SEM (n = 5 transfections per group). Kruskal-Wallis test $p < 0.0001$; and Dunn's multiple comparisons test are indicated as * $p<0.05$, ** = $p<0.01$ vs. K758R and # $p<0.05$, ## = $p<0.01$ vs. GFP. (**B**) Animals were injected unilaterally in the right SNc with AAV vectors expressing wt PLD2, Y179F, R180K, K758R or GFP. 2 weeks postinjection, nigral tissue from both sides of the brain was collected and PLD activity was measured in both the injected and uninjected sides of individual animals. Results are expressed as % PLD activity on the injected side over the uninjected side (which has a PLD activity of 100%), as mean ± SEM (n = 4–6 animals per group). One-way ANOVA statistics were as follow: $F[4,15] = 6.369$, $p<0.01$; and Turkey's post hoc results are indicated as * = $p<0.05$ vs K758R, ** = $p<0.01$ vs K758R and # = $p<0.05$ vs GFP.

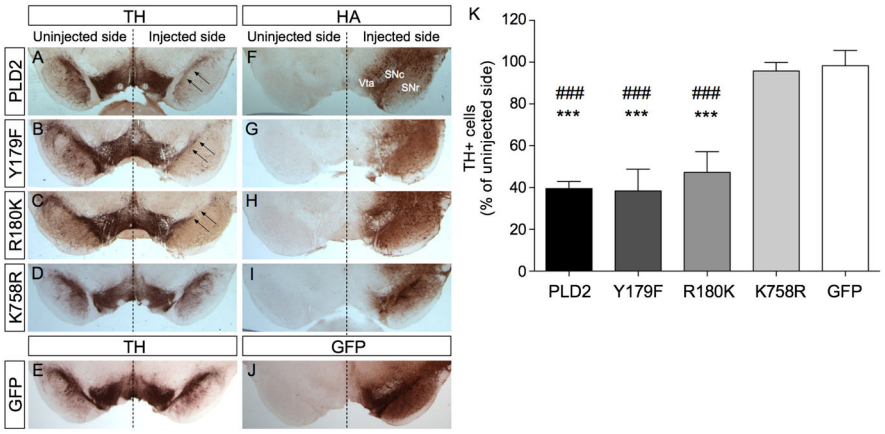

Fig. 3. Number of TH$^+$ neurons in the SNc after overexpression of wt PLD2 and mutants

Photomicrographs of nigral sections from rats 4 weeks after AAV injections containing PLD2, Y179F, R180K, K758R or GFP, stained for TH (A–E), HA (F–I) and GFP (J). Arrows mark the substantia nigra pars compacta (SNc). PLD2, Y179F, R180K, K758R proteins can be detected with the anti-HA antibody because they have the flag HA epitope inserted at the N terminus of the protein. Unbiased stereology estimation of nigral TH$^+$ cells of the SNc was measured (K) and results are expressed as % of TH$^+$ cells on the injected side over the uninjected side, as mean ± SEM (n = 5–6 animals per group, one single data point per animal). One-way ANOVA statistics are: $F[4,21] = 17.41$, $p < 0.0001$; and Turkey's post hoc results are indicated as *** = $p<0.001$ vs K758R and ### = $p<0.001$ vs GFP. Vta, ventral segmental area. SNr, substantia nigra pars reticulate.

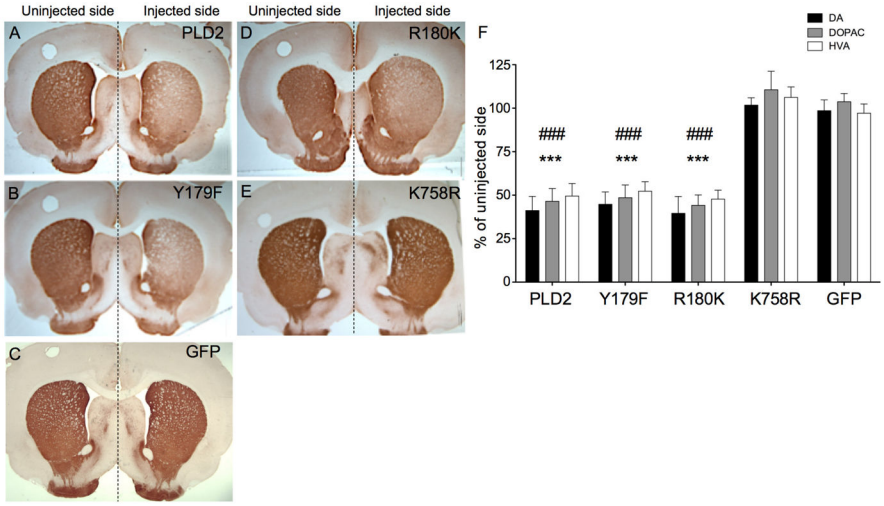

Fig. 4. TH expression and dopamine, DOPAC and HVA levels in the striatum with overexpression of wt PLD2 and PLD2 mutants

Photomicrographs of striatal sections stained for TH (A–E) from rats 4 weeks after injections in the SNc of AAV containing wt PLD2 (A), Y179F (B), R180K (D), K758R (E) or GFP (C). Tissue extractions of striatum were processed as described in Materials and Methods and DA, DOPAC and HVA levels were measured (F). The results are expressed as % of DA, DOPAC and HVA on the injected side over the uninjected side, as mean ± SEM (n = 5 animals per group). One-way ANOVA statistics were: $F[4,20] = 19.35$, $p < 0.0001$ for DA, DOPAC and HVA; Turkey's post hoc results are indicated as *** = $p<0.001$ vs K758R and ### = $p<0.001$ vs GFP.

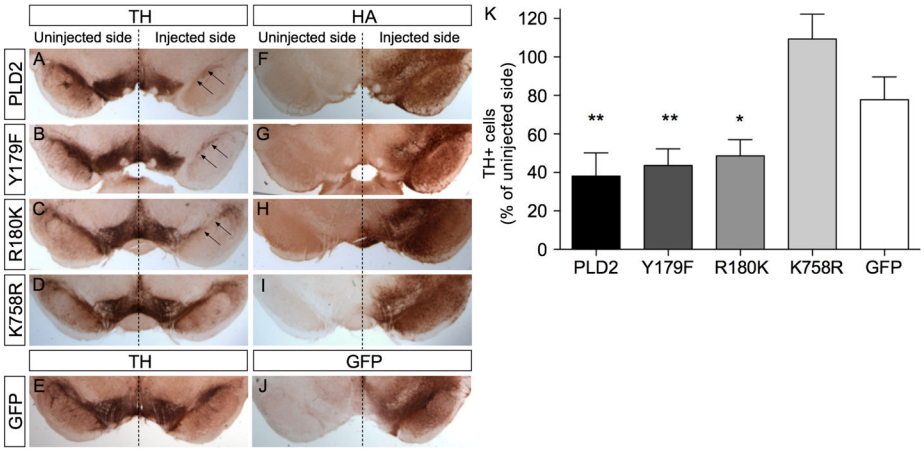

Fig. 5. Number of TH$^+$ neurons in the SNc after longer overexpression of wt PLD2 and mutants

Photomicrographs of nigral sections from rats 10 weeks after AAV injections containing wt PLD2, Y179F, R180K, K758R or GFP, stained for TH (A–E), HA (F–I) and GFP (J). Unbiased stereology estimation of nigral TH$^+$ cells of the SNc was measured (K) and results are expressed as % of TH$^+$ cells on the injected side over the uninjected side, as mean ± SEM (n = 4–8 animals per group, one single data point per animal). One-way ANOVA statistics were: F[4, 20] = 7.31, p= 0.0008; and Turkey's post hoc results are indicated as *, ** = p<0.05, and p<0.01, respectively vs. K758R.

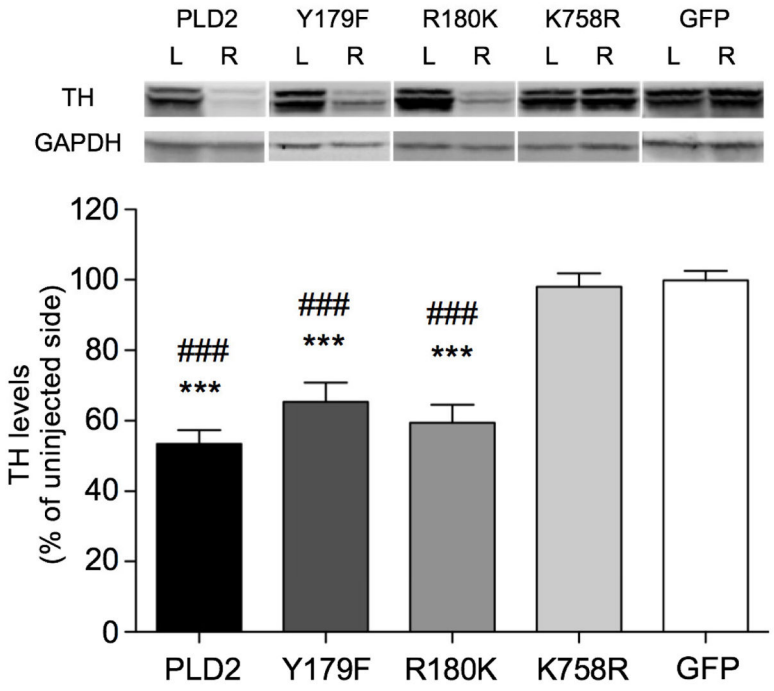

Fig. 6. TH expression in the striatum after longer overexpression of wt PLD2 and mutants

Striatal tissue from 10-week animals was extracted and pooled and then immunoblotted with antibody TH. GAPDH was used as control of loading. Results are expressed as % of TH levels on the injected side over the uninjected side, normalized with GAPDH levels, as mean ± SEM (n = 4–8 animals per group). One-way ANOVA statistics were as follow: $F[4,21] = 25.75$, $p < 0.0001$; and Turkey's post hoc results are indicated as *** = $p < 0.001$ vs. K758R and ### = $p < 0.001$ vs. GFP.