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Impaired brain development and reduced cognitive function in phospholipase D-deficient mice

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HIGHLIGHTS

- Genetic deletion of phospholipase D 1 or 2 delays brain development in mice.
- Cognitive function is reduced in adult PLD-deficient mice.
- PLD deficiency impairs release of acetylcholine after behavioral stimulation.
- Disruption of PLD signaling may contribute to fetal alcohol syndrome and Alzheimer' disease.

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ABSTRACT

The phospholipases D (PLD1 and 2) are signaling enzymes that catalyze the hydrolysis of phosphatidylcholine to phosphatidic acid, a lipid second messenger involved in cell proliferation, and choline, a precursor of acetylcholine (ACh). In the present study, we investigated development and cognitive function in mice that were deficient for PLD1, or PLD2, or both. We found that PLD-deficient mice had reduced brain growth at 14–27 days *post partum* when compared to wild-type mice. In adult PLD-deficient mice, cognitive function was impaired in social and object recognition tasks. Using brain microdialysis, we found that wild-type mice responded with a 4-fold increase of hippocampal ACh release upon behavioral stimulation in the open field, while PLD-deficient mice released significantly less ACh. These results may be relevant for cognitive dysfunctions observed in fetal alcohol syndrome and in Alzheimer' disease.

1. Introduction

The phospholipases D (PLD) are ubiquitous enzymes which catalyze the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. Phosphatidic acid is a lipid second messenger which regulates cytoskeletal organization and vesicular trafficking and is involved in cell proliferation. Choline is a precursor of acetylcholine (ACh) synthesis and of choline-containing phospholipids such as PC and sphingomyelin. The two major mammalian isoforms of PLD are PLD1 and PLD2. PLD1 is located in the perinuclear region, is activated by small GTPases such as ARF and Rho and participates in budding and fusion of secretory vesicles and in stress fiber formation. In contrast, PLD2 is located at the cellular membrane, shows high basal activity, is regulated by tyrosine kinases and protein kinase C and participates in receptor endocytosis (reviewed in [20]). PLD1 and 2 are capable of transphosphatidylation, a reaction in which PC is transformed into phosphatidylalcohols (e.g., phosphatidylethanol) when alcohols (e.g. ethanol) are present; thus, the PLD signaling pathway is suppressed in the presence of alcohols because PA formation is reduced [1,14].

Numerous studies have implicated PLD activation in the control of cell proliferation [9]. Importantly, several lines of evidence support a role of PLDs in cognitive function or dysfunction. Previous work of our group and others demonstrated a role for PLD in early

Abbreviations: ACh, acetylcholine; ACSF, artificial cerebrospinal fluid; MRI, magnetic resonance imaging; ORT, object recognition task; PA, phosphatidic acid; PC, phosphatidylcholine; PLD, phospholipase; DSRT, social recognition task.

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development of the brain. In fact, growth factors stimulate PLD activity in neurons and astrocytes which results in cell proliferation [19,26]. Astrocytes interact closely with neurons during the brain growth spurt enabling neuritogenesis and formation of synapses, two processes in which PLDs are involved [13]. Accordingly, interruption of PLD signaling, e.g. by alcohols, may inhibit normal brain development [14] by inhibiting the formation of the lipid messenger, phosphatidic acid; this mechanism may be partly responsible for ethanol toxicity to the fetus ("fetal alcohol syndrome") [1].

There is also evidence that links PLD to neurotransmitter release, especially in cholinergic neurons. In whole cells, PLD activity can be activated by muscarinic receptors [14]. In synaptosomes, PLD is activated by depolarization and by calcium influx [22,28]. Both PLD isoforms may be involved in neurotransmitter release. In one study, catalytically inactive PLD1 mutants (K898R) reduced ACh release in Aplysia neurons by reducing the number of active presynaptic releasing sites [12]. In another study, PLD2 was responsible for the generation of choline for ACh synthesis, and downregulation of PLD2 led to reduce ACh release in cholinergic neurons [29].

Another line of investigation links PLD activities to Alzheimer' disease (AD), which is also characterized by cognitive deficits and by widespread cholinergic dysfunction. PLD activity was reduced in cells with AD-like mutations, and overexpression of PLD1 in these cells corrected trafficking alterations of the amyloid precursor protein [2]. In contrast, amyloid β toxicity in cell cultures was promoted by PLD2 activity [18]. In addition, a third PLD form ("PLD3") was associated with Alzheimer' disease in a recent genome-wide association study [5], but further characterization of this new isoform is required to address its potential role in lipid signaling.

In the present study, we made use of recently developed mouse models that are deficient in PLD1 or PLD2. In light of the previous reports summarized above, we investigated brain growth in juvenile PLD-deficient mice and tested cognitive function and cholinergic activity in adult mice *in vivo*. We found that characteristic impairments can be seen in both PLD-deficient strains, with stronger effects in PLD2-deficient mice.

2. Materials and methods

2.1. Materials

Chemicals for artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$) were obtained from Sigma–Aldrich (Seelze, Germany). Neostigmine bromide was obtained from Acros Organics (Thermo Fisher Scientific, Schwerte, Germany) and scopolamine bromide from TCI Europe (Eschborn, Germany). Ketamine/xylazine was prepared with Rompun® 2% (Bayer Co.) and Ketavet® 100 mg/ml (Pfizer Co.).

2.2. Animals

Gender-mixed *Pld1*$^{-/-}$, *Pld2*$^{-/-}$ mice (generated on a B6 background) and wild-type controls [7,27] were housed in a facility with controlled temperature and humidity and a day/night cycle of 12/12 h. They had free access to food and water. All animal experiments were performed in agreement with EU directive 2010/63 and were registered with the local animal committee (Regierungspräsidium Darmstadt, Germany).

2.3. Magnetic resonance imaging (MRI)

The MRI study was performed in a 3 T Magnetom® Trio (Siemens, Erlangen, Germany) with circular polarized wrist coil. The mice were assessed once a week over a time period of 5 weeks beginning at the age of P7. The litter size of all groups was 6–8 pups and the mothers had free access to food and water. Prior to MRI, pups were anesthetized with ketamine/xylazine (70/7 mg/kg body weight i.p.) and kept warm throughout the entire measurement. The brain size of the pups was measured by MRI in 19–20 transversal T2-weighted spin echo sequences with a slice thickness of 0.9 mm without gap and an in-plane resolution of 0.2 × 0.2 mm. The measurements lasted around 9 min. Resulting DICOMs were analyzed by marking the brain area in each slice using free MRIcro software. The brain volume was calculated by addition of brain areas (cortices) in all slices.

2.4. Behavioral testing

3–4 months old mice were placed in the behavioral testing room at least 1 week before the experiments took place. Behavioral testing was performed during their active time (night cycle) using red light enabling observation. All mice moved freely and showed no sign of fear or stress during the experiments. Mice which did not interact with objects (object recognition test) and mice which fell off the platform (passive avoidance) several times were excluded (one each in control and *Pld1*$^{-/-}$ group and three in the *Pld2*$^{-/-}$ group). After each trial, objects and environment were cleaned to eliminate odor cues.

The social recognition task [10] tests short-term memory [24]. In an open field of 75 × 43 × 20 cm, each mouse was presented for 5 min with an empty cage and a second cage containing an unknown mouse of the same gender. Contacts with the mouse and the empty cage were scored. After a break of 15 min, the mouse was confronted for 5 min with the familiar mouse of the first session in one cage and a novel mouse (same gender) in the other cage and the contacts with both mice were counted. Learning and memory were scored using the level of discrimination (LD). It was calculated by the formula $(b-a)/(b+a)$, where a and b are the contacts with the familiar and novel mouse, respectively.

The object recognition task (ORT; [8]) is a test for long-term memory. In a grey box of 45 × 30 × 15 cm, each mouse was presented for 5 min with two identical objects (cylinders) and contacts with both objects were scored. On the next day, mice were confronted with two familiar and an additional novel object (pyramid) and contacts with each object were counted again. The LD was calculated as $(b-a)/(b+a)$, in which "a" is the mean of contacts with the familiar objects and "b" the contacts with the novel object.

The passive avoidance task [4] was used to assess learning based on aversive stimuli. Briefly, mice were put on a small, 20 cm high platform in front of an opening to a dark box of 20 × 20 × 20 cm. The platform was lighted to further facilitate entering into the box. A grid at the bottom of the box transmitted a mild foot shock (0.5 mA, 1 s), delivered immediately when the mouse entered the box. The time till entering the box was measured (s). On day 2, the mouse was placed on the platform again and the time till entering the box was measured again (s). The experiment was stopped when mice stayed on the platform longer than 3 min.

2.5. Microdialysis

Mice were anesthetized with isoflurane (induction dose, 4%; maintenance dose, 1–1.5% v/v) in synthetic air and placed in a stereotaxic frame. Self-made, I-shaped, concentric dialysis probes with an exchange length of 2 mm were implanted in the right ventral hippocampus using the following coordinates (from bregma): anterior–posterior, −2.7 mm; lateral, −3.0 mm; dorsoventral, −3.8 mm [19]. Mice were allowed to recover overnight, and experiments were carried out on the following day in awake, freely moving animals [17]. The microdialysis probes were perfused with artificial cerebrospinal fluid (aCSF) supplemented with 0.1 μM neostigmine bromide. The perfusion rate was 1 μl/min, and efflux from the microdialysis probe was collected in intervals of 15 min.

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