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ETOH inhibits embryonic neural stem/precursor cell proliferation via PLD signaling

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ABSTRACT

While a mother's excessive alcohol consumption during pregnancy is known to have adverse effects on fetal neural development, little is known about the underlying mechanism of these effects. In order to investigate these mechanisms, we investigated the toxic effect of ethanol (ETOH) on neural stem/precursor cell (NSC) proliferation. In cultures of NSCs, phospholipase D (PLD) is activated following stimulation with epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2). Exposure of NSCs to ETOH suppresses cell proliferation, while it has no effect on cell death. Phosphatidic acid (PA), which is a signaling messenger produced by PLD, reverses ETOH inhibition of NSC proliferation. Blocking the PLD signal by 1-butanol suppresses the proliferation. ETOH-induced suppression of NSC proliferation and the protective effect of PA for ETOH-induced suppression are mediated through extracellular signal-regulated kinase signaling. These results indicate that exposure to ETOH impairs NSC proliferation by altering the PLD signaling pathway.

Fetal alcohol syndrome (FAS) is a major consequence of a mother's excessive alcohol consumption during pregnancy. FAS is characterized by three peculiar symptoms: growth retardation, craniofacial abnormalities, and neurological dysfunctions [1]. The development of the central nervous system (CNS) involves stepwise events consisting of proliferation, migration, differentiation, and programmed cell death of immature cells. Among these events, the proliferation of immature neural cells appears to be sensitive to ETOH exposure.

Considering the deleterious effects of alcohol on CNS development, neural stem/precursor cells (NSCs) could provide a suitable in vitro model for analyzing alcohol toxicity. NSCs are defined as self-renewing and multipotent cells that can generate all major CNS cell types: neurons, astrocytes, and oligodendrocytes. A number of intrinsic and extrinsic factors, such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2), control NSC development in vitro and in vivo [2]. Previous studies have reported that alcohol exposure disturbs the cell proliferation in the ventricular zone where NSCs reside and proliferate rapidly during the embryonic stage [3]. However, the molecular mechanisms of this effect have not been clarified.

The lipid-metabolizing enzyme phospholipase D (PLD) is a possible candidate for the vehicle by which ETOH exposure inhibits NSC proliferation. In physiological conditions, PLD hydrolyzes phosphatidylcholine (PC), which is one of the membrane phospholipids. This enzymatic reaction releases choline and produces phosphatidic acid (PA), which is known to play several physiological roles as a second messenger. PLD is widely distributed in higher eukaryotic organisms and activated by a variety of extracellular stimuli including growth factors, hormones, cytokines, neurotransmitters, and adhesion molecules, whose signals are transduced through a G protein-coupled receptor or receptor tyrosine kinases [4]. An important role of the PLD signal is to promote cellular proliferation [5]. In previous studies, it has been reported that PLD is activated by a variety of mitogens, PDGF [6], FGF [7], EGF [8], and an insulin-like growth factor [9], suggesting that the activated PLD signal is involved in promoting cellular proliferation. It is known that primary alcohols, such as ETOH and 1-butanol, have an antagonistic effect on the PLD signal. In the presence of primary alcohols, the PLD signal is inhibited by competitively diverting the generation of PA to non-physiological phospholipids [10]. In the developing CNS, two PLD isoforms, PLD1 and PLD2, have been found in both neurons and glial cells and these isoforms differ somewhat in their subcellular localization and function [11–14]. However, little is known about the role of PLD in NSC development.

Materials and methods

Cell isolation and culture. Ganglionic eminences were dissected from C57BL/6 mice on embryonic day 14.5 (E14.5). The animals were killed in accordance with institutional guidelines for the Care and Use of Laboratory Animals of the National

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Research Institute for Child Health and Development. Tissues were placed into Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (Invitrogen) and dissociated cells were re-suspended into a neurosphere medium DMEM/F-12 (Invitrogen) supplemented with EGF (Peprotech, 20 ng/ml), FGF2 (Peprotech, 20 ng/ml), and B27 (Invitrogen, 1:50) unless otherwise indicated. The cells were then cultured in 25 cm^2 tissue culture flasks (Iwaki) at a density of 1×10^5 cell/ml for 5–7 days. The proliferation of neurospheres was assessed by measuring the diameters of neurospheres using Zeiss axiovision software (Zeiss).

TdT-mediated dUTP nick-end labeling (TUNEL) analysis. Cryostat sections of neurospheres were prepared for TUNEL analysis as follows: Neurospheres were collected into 15-ml tubes and fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). After fixing, the neurospheres were embedded in Tissue Tek O.C.T. compound (Sakura Finetechnical) and cryosectioned at 10 μm. TUNEL staining was carried out using an in situ cell death detection kit, TMR Red (Roche), following the manufacturer's instructions. The number of DAPI-labeled nuclei was counted and compared to the number of TUNEL-positive cells.

RT-PCR analysis. Total RNA was prepared from neurospheres after 7 days in culture using the RNA extraction kit ISOGEN (Nippon Gene), and 1 μg of RNA was transcribed using Superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. The resulting cDNA was subjected to PCR using an Ex-Taq kit (Takara Bio) as described elsewhere [15]. The sense and antisense primers, respectively, were as follows: GAPDH, 5′-GGTCATCATCTCCGCCCCTTC-3′ and 5′-CCACCACCCTGTTGCTGTAG-3′; PLD1, 5′-ATGGAATCCTTAAGCCTCAAG-3′ and 5′-CAC CTGATATCTCAACTCGTG-3′; PLD2, 5′-AGCAAGAGGACGCTGATGC-3′ and 5′-CAAAGG TGTCTTATACCTGGC-3′.

In vitro PLD assay. Phosphatidylinositol 4,5-bisphosphate (PIP_2)-dependent PLD activity was assayed by measuring choline release from phosphatidylcholine. Neurospheres cultured for 5 days were deprived of EGF and FGF2 for 24 h. Twenty-four hours later, neurospheres were exposed to EGF (20 ng/ml) or FGF2 (20 ng/ml) or a combination of both growth factors for 5, 30, 60, and 120 min. After exposure, neurospheres were collected by centrifugation and washed with ice-cold PBS. They were then homogenized on ice with a PLD lysis buffer (20 mM HEPES [pH 7.6], 10% glycerol, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol) supplemented with a protease mixture (5 μg/ml luepoptin, 5 μg/ml aprotinin, and 1 mM 4-[2-aminoethyl]-benzene-sulfonylfluoride) with a 27-gauge needle. After centrifugation at 500*g* for 10 min, the supernatant was centrifuged at 120,000*g* at 4 °C for 30 min to separate the membrane and the cytosolic fraction. The resulting pellets were resuspended with a PLD lysis buffer, and 1 μg of protein was used for the assay. The reaction was carried out at 37 °C for 15 min in a 60-μl assay mixture containing the PLD preparation, 10 μl of the PLD assay buffer (250 mM HEPES [pH 7.2], 15 mM EGTA, 6 mM dithiothreitol, 400 mM KCl, 18 mM $MgCl_2$, and 12 mM CaC_2), and 10 μl of phospholipid vesicles (dioleoylphosphatidylethanolamine [SIGMA], dipalmitoylphosphatidylcholine [SIGMA], and PIP_2 [Wako] at a molar ratio of 16:1:1.4) and dipalmitoylphosphatidyl-[*methyl*-^{3}H] choline [PerkinElmer]; 0.5 μCi/assay). The reaction was then terminated by adding 1 ml of chloroform/methanol/HCl (50:50:0.3) and 140 μl of 5 mM EGTA in 1 N HCl. The mixture was separated into two phases by centrifugation at 13,000*g* for 5 min. The amount of [*methyl*-^{3}H] choline in the aqueous phase was quantified by liquid scintillation counting.

Assay for MAP/extracellular signal-regulated kinase (ERK) activity and Western blot. The neurospheres cultured for 3 days in a neurosphere medium were used for the assay of MAPK activity. Some neurosphere cultures were treated with ETOH (200 or 300 mM) or 1,2-dioctanoyl-sn-glycerol 3-phosphate sodium salt (PA, 1 μg/ml, SIGMA) for 10 min. To stop MAPK activity, the mitogen-activated protein kinase kinase (MEK)-specific MAPK inhibitor U0126 (10 μM, Promega) was added to the medium for 10 min prior to ETOH or PA treatment. After individual cultures were prepared, neurospheres were collected by centrifugation and washed with ice-cold PBS. They were then sonicated on ice in a standard lysis buffer and boiled in an SDS–PAGE sample buffer [15]. Proteins (15 μg) were separated by 12.5% SDS–PAGE gels and transferred onto PVDF membranes. The blots were incubated with a polyclonal rabbit anti-p44/42 MAP kinase antibody (1:1000, Cell Signaling) and a polyclonal rabbit anti-phospho-p44/42 MAP kinase antibody (1:1000, Cell Signaling) as described elsewhere [15].

Results and discussion

ETOH exposure at low doses inhibits NSC proliferation without inducing apoptosis

In order to investigate the effects of ETOH on fetal neural development, we used an in vitro culture of neurospheres. In the neurosphere medium supplemented with EGF and FGF2, NSCs grew on the surface of the medium and formed round cell aggregations, i.e., neurospheres (Fig. 1B). Since sufficient concentrations of many teratogens are known to induce apoptosis, we first assessed the ability of ETOH exposure to induce apoptosis in NSCs. We prepared cryostat sections of neurospheres both positive and negative for ETOH exposure and carried out TUNEL assays to detect apoptotic cells (Fig. 1Aa–e and C). TUNEL-positive cells were rarely present in control (ETOH-exposure negative) neurospheres (<0.1%), and there was no significant increase in the number of TUNEL-positive cells in neurospheres treated with ETOH at concentrations from 50 to 200 mM. However, in neurospheres treated with 400 mM ETOH, TUNEL-positive cells were significantly increased ($P < 0.001$) (Fig. 1Ae and C).

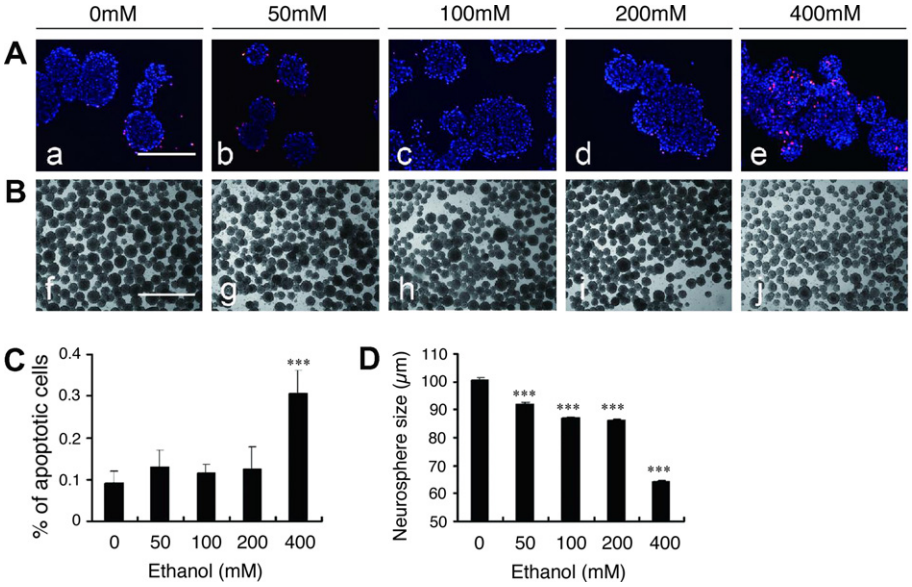

Fig. 1. Effects of ETOH on cell survival, EGF- and FGF2-stimulated proliferation, and differentiation into neurons. (A) Apoptotic cells (red) were stained by the TUNEL method on 10 μm cryostat sections of neurospheres. Nuclei were counterstained with DAPI (blue). (B) Photomicrographs show that ETOH exposure suppresses NSC proliferation in neurosphere media containing EGF- and FGF2. NSC proliferation was evaluated by measuring the diameter of neurospheres. (C,D) The experiments presented in (A,B) were quantified. The data are displayed as means ± SD. Asterisks indicate statistical significance. $^{***}P < 0.001$ vs. control (Student's *t*-test). Scale bars in (Aa), 20 μm for (Aa–e); in (Bf), 200 μm for (Bf–j).

Next, the effect of ETOH on NSC proliferation was evaluated by measuring the diameter of neurospheres positive or negative for 7-day ETOH exposure (Fig. 1Bf–j, D). The mean neurosphere size was 100.5 ± 0.7 μm in the ETOH-negative neurospheres. Treatment with ETOH from 50 to 400 mM significantly reduced the neurosphere size, indicating that ETOH has an effect on the proliferation of NSCs and that a low concentration of ETOH (up to 200 mM) can inhibit cell proliferation without inducing apoptosis.

PLD1 and 2 are expressed in NSCs and activated by EGF and/or FGF stimuli

Previous studies have reported that PLD is expressed in neurons, astrocytes and oligodendrocytes [11], whereas the expression of PLD in NSCs has not been reported. Therefore, we analyzed the expression of PLD1 and PLD2 in NSCs by RT-PCR. As shown in Fig. 2A, both PLD isoforms, PLD1 and PLD2, were expressed in cells from neurospheres. The embryonic telencephalon at E14, in which NSCs proliferate and differentiate, also expressed PLDs.

We next examined PLD activity in response to EGF and FGF2, which are well-known mitogens for NSCs, using an in vitro PLD assay. After mitogen starvation for 24 h, NSCs were treated with EGF (20 ng/ml) and/or FGF2 (20 ng/ml) for 5, 30, 60, and 120 min. As shown in Fig. 2B, PLD activity was significantly increased within 30 min of treatment with these growth factors. After 60 min of EGF treatment, the maximal PLD activity was attained (1.7-fold above the basal activity; $P < 0.05$). Thereafter, the activity was slightly decreased but still sustained at a significant level relative to the control ($P < 0.05$). In contrast, FGF2 stimulation resulted in higher PLD activity than EGF treatment at all time points. A significant increase in PLD activity was achieved after 30 min of FGF treatment (1.9-fold above the basal activity; $P < 0.05$). Even after 120 min of FGF stimulation, PLD activity was significantly increased (2.4-fold above the basal activity; $P < 0.05$). The combined effect of EGF and FGF2 on PLD activity was additive. PLD activity was significantly increased by EGF/FGF2 treatment within 5 min (2.1-fold above the basal activity; $P < 0.05$) and reached 3-fold after 120 min. Thus, PLD activity is stimulated by mitogens in NSCs, as well as in neurons [11], astrocytes [16] and oligodendrocytes [17].

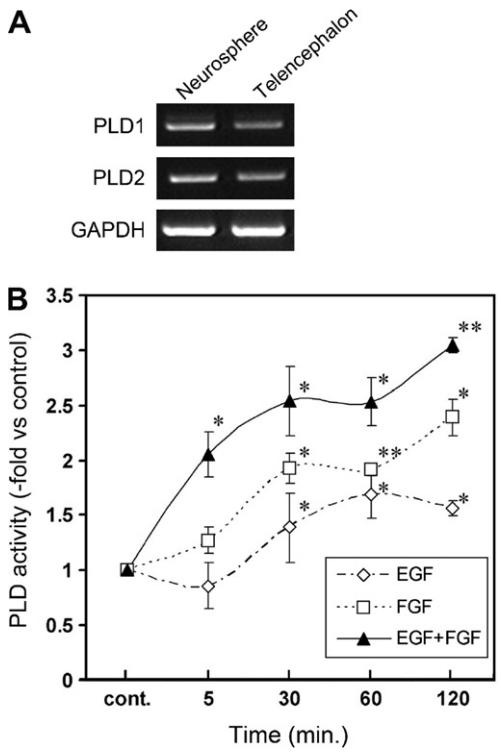

Fig. 2. Detection of PLD genes and upregulation of PLD enzymatic activity induced by EGF and/or FGF2 stimuli in NSCs. (A) RT-PCR analysis to detect PLD1 and PLD2 in neurospheres maintained for 7 days (upper panel) and in the telencephalon in E14 mouse (middle panel). (B) Time course of PLD activity induced by EGF and FGF2 stimuli. The basal PLD activity was defined as 1. The data are shown as changes of the basal PLD activity. Asterisks indicate statistical significance. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control (Student's *t*-test). Error bars indicate SD.

Exogenous PA can prevent the adverse effect of ETOH exposure on NSC proliferation

In previous studies, it has been reported that the mitogen-stimulated PLD signal promotes proliferation in various types of cells [5] and that the PLD-mediated proliferation is inhibited by ETOH exposure [16]. Taking these previous findings into account, we hypothesized that ETOH could impair NSC proliferation by altering the PLD signal. To study this hypothesis, we first examined whether the enhancement of the PLD signal can promote NSC proliferation. To stimulate the PLD signal, we added exogenous PA, a major messenger molecule generated by PLD, into neurosphere culture medium. Treatment with PA (1 or 2 μg/ml) for 7 days significantly increased the neurosphere size (Fig. 3A, 1 μg/ml; $P < 0.05$, 2 μg/ml; $P < 0.001$), suggesting that the PLD-PA signal is one of the pathways that positively regulates NSC proliferation under the normal conditions.

If ETOH inhibits NSC proliferation by disturbing the PLD signal, enhancement of PLD activity could mitigate the adverse effect of ETOH on NSC proliferation. To study the hypothesis, we added PA to the neurosphere culture medium in the presence of ETOH (200 mM). As expected, the addition of PA increased the neurosphere size in a dose-dependent manner, with a plateau at 0.05 μg/ml (Fig. 3B). When 0.05 μg/ml of PA was added, the reduction in the neurosphere size caused by ETOH exposure was completely reversed. These results suggest that ETOH's suppression of NSC proliferation may result from a decrease in PLD-signal-catalyzed PA production.

Blockade of endogenous PA production by 1-butanol as also inhibits NSC proliferation

To confirm that the suppression of NSC proliferation by ETOH exposure is a consequence of reduced PA production (catalyzed by PLD), we examined the effect of blocking endogenous PA production on NSC proliferation by using primary alcohols other than ETOH. PLD catalyzes a transphosphatidylation reaction using water or primary alcohols, such as ETOH and 1-butanol. As a result of this reaction, PA, phosphatidyl ETOH (PEt) and phosphatidylbutanol (PBt) are generated from water, ETOH, and 1-butanol, respectively. It has been reported that primary alcohols are preferentially used over water by 1000-fold or more [18] and interfere with PLD-signal-catalyzed PA production. However, tertiary alcohol cannot be substituted for water [10]. Thus, we compared the effect of isomeric alcohols, primary alcohol (1-butanol) and tertiary alcohol (*tert*-butanol), on NSC proliferation. As shown in Fig. 3C and D, 1-butanol strongly suppressed the formation of neurospheres. Treatment with 25 mM of 1-butanol decreased the neurosphere size (Fig. 3Cb). In cultures treated with 50 and 100 mM of 1-butanol, the formation of neurospheres was completely inhibited (Fig. 3Cc and Dd). In contrast, *tert*-butanol had a lesser effect than 1-butanol (Fig. 3Ce–g). Treatment with *tert*-butanol also decreased the neurosphere size, but neurospheres formed at all concentrations tested. Thus, 1-butanol (primary alcohol) suppressed NSC proliferation more strongly than *tert*-butanol. These results further support the idea that ETOH exposure inhibits NSC proliferation by disturbing the PLD signal.

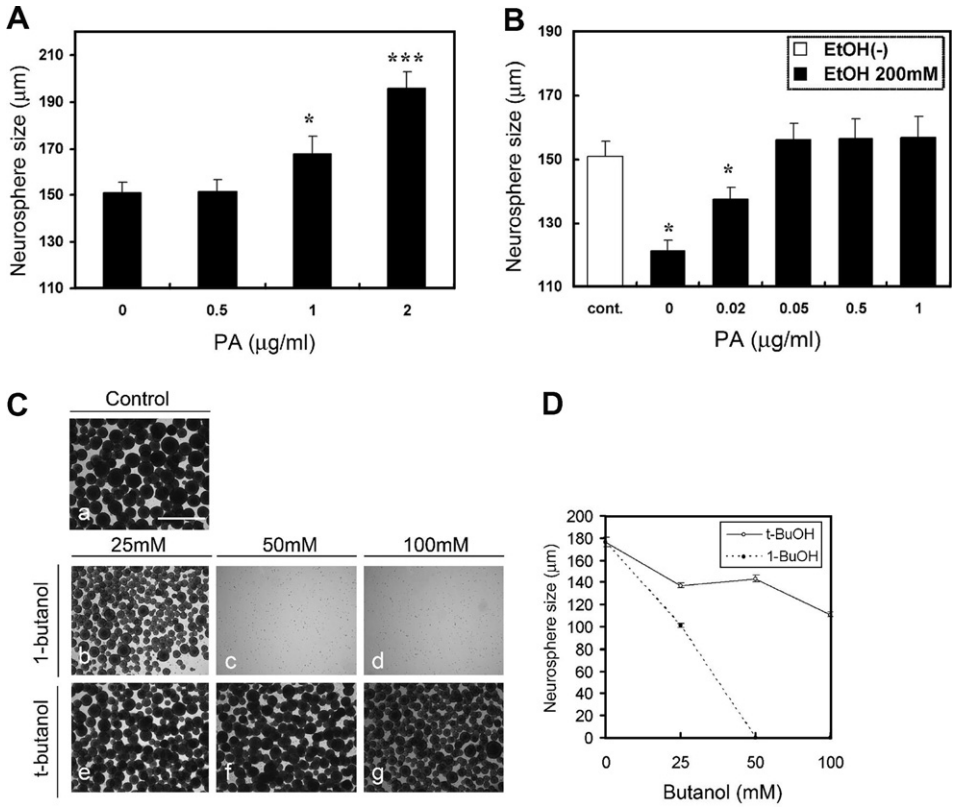

Fig. 3. Effects of PA and 1-butanol on NSC proliferation. Exogenous PA facilitates NSC proliferation and can reverse ETOH-induced suppression of NSC proliferation (A, B). NSC proliferation was evaluated by measuring the neurosphere size 7 days after the addition of exogenous PA in the absence (A) or presence of 200 mM of ETOH (B). The experiments were quantified and indicated as means ± SD. Asterisks indicate statistical significance. $^{*}P < 0.05$; $^{***}P < 0.001$ vs. control (Student's *t*-test). (C) Primary alcohol (1-butanol) suppresses NSC proliferation more strongly than tertiary alcohol (*tert*-butanol). Different isomeric alcohols were added to the neurosphere culture medium. (C) Photomicrographs showing different effects of 1-butanol and *tert*-butanol exposure on NSC proliferation. (D) The experiments were quantified and indicated as means ± SD. Scale bar in (Ca), 200 μm for (Ca–g).

Elimination of the PLD signal by ETOH exposure inactivates the MAP/ERK pathway and yields reduced NSC proliferation

The question of which downstream effector is involved in the action of ETOH on the inhibition of NSC proliferation caused by disturbance the PLD signal remains to be answered. Although it has been shown that the PLD signal interacts with several downstream targets [5], we examined the MAP/ERK pathway, which is known to be inactivated by ETOH exposure, leading to the suppression of agonist- or mitogen-induced proliferation in non-neural cells [19].

First, we investigated whether ETOH exposure inhibited the MAP/ERK pathway. To address this, we examined the phosphorylation of ERK1/2 in neurospheres treated with or without ETOH and/or PA. We also used a protein kinase inhibitor U0126 (5 μM), which specifically blocks the activities of the mitogen-activated extracellular signal-regulated kinase kinase (MEK) that acts upstream of ERK1/2. In the control culture, an endogenous phosphorylated ERK1/2 was detectable by Western blot (Fig. 4A, lane 1 in the upper panel). PA treatment slightly increased phosphorylated ERK1/2, especially ERK2 (Fig. 4A, lane 4 in the upper panel). ETOH exposure (200 and 300 mM) decreased the phosphorylation level of ERK1/2, especially ERK2 (Fig. 4A, lanes 2, 3 in the upper panel), and enhancement of the PLD signal by adding PA (1 μg/ml) markedly reversed the inactivation of ERK1/2 (Fig. 4A, lanes 5, 6 in the upper panel). The inclusion of U0126 reduced both endogenous (Fig. 4A, lane 7 in the upper panel) and PA-induced (Fig. 4A, lanes 8, 9, 10 in the upper panel) ERK1/2 phosphorylation. The total ERK levels did not change after any culture treatment (Fig. 4A, lower panel).

We then investigated whether U0126 could abolish the ability of PA to reverse the ETOH-induced suppression of NSC proliferation. Treatment with U0126 alone for 7 days significantly decreased the neurosphere size (Fig. 4B, $P < 0.001$), suggesting that the ERK signaling pathway contributes to NSC proliferation under normal conditions. When U0126 was applied to the neurosphere medium with ETOH (200 or 300 mM) and PA, PA failed to reverse the reduction of the neurosphere size (Fig. 4B), indicating that PA's ability to mitigate ETOH's inhibition of NSC proliferation requires the activation of ERK. These results suggest that the disturbance of the PLD signal by ETOH exposure causes the inactivation of the MAP/ERK pathway, ultimately suppressing NSC proliferation.

Significance of the PLD signal in neurological abnormalities in FAS

We can draw three conclusions based on the present findings: first, treating NSCs with low doses of ETOH results in the suppression of EGF- and FGF2-induced cell proliferation without inducing apoptosis or altering neuronal differentiation. Second, ETOH exposure alters the PLD signal by interfering with PA production, thereby leading to the suppression of NSC proliferation. Third, the PLD signal via MAPK/ERK is inhibited by ETOH, which results in suppressed proliferation of NSCs. Our findings indicate that the PLD signal plays a crucial role in NSC proliferation and that ETOH's effect on the PLD signal could contribute to the neurodevelopmental abnormalities observed in patients with FAS.

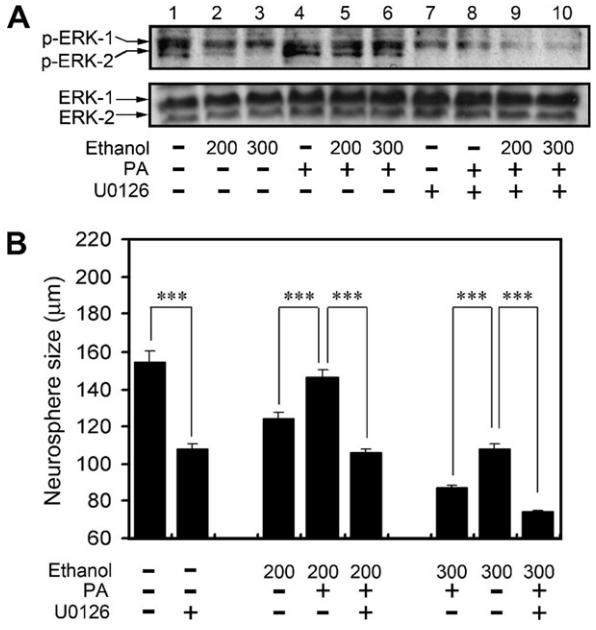

Fig. 4. Interplay of the antiproliferative ETOH action on NSCs, the PLD signal, and MAPK/ERK activity. NSCs were treated with ETOH (200 or 300 mM), exogenous PA, and MEK inhibitor U0126. (A) The cells were subjected to Western blot analysis using either the anti-phosphorylated ERK antibody (p-ERK-1/2, upper panel) or the anti-ERK (ERK-1/2, lower panel) antibody. The data are representative results of an experiment that was repeated two times. (B) The histogram compares NSC proliferation in individual cultures. The experiments were quantified and are indicated as means ± SD. Asterisks indicate statistical significance. $^{***}P < 0.001$ vs. control (Student's *t*-test).

Alcohol exposure during different embryonic stages produces different outcomes, reflecting a stage-specific vulnerability to alcohol. Microencephaly is one of the most characteristic consequences of ETOH exposure in utero, observed in more than 80% of FAS children [20]. A critical period of ETOH-induced microencephaly occurs during the third trimester of pregnancy in humans [20]. During this period, the NSCs are generated and rapidly proliferate in the developing brain. The reduction in NSC proliferation caused by ETOH exposure might lead to a reduction in the final number of cells in the mature brain.

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