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Ceramide is involved in alcohol-induced neural proliferation

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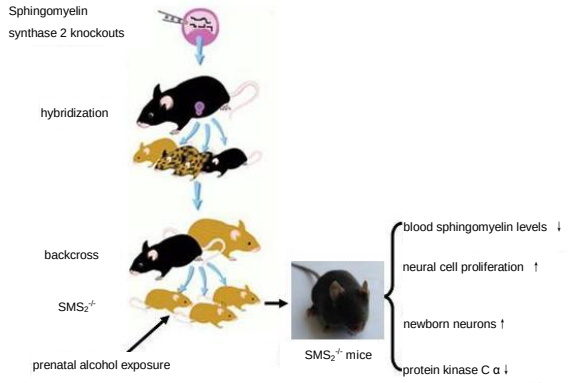
Graphical Abstract



Abstract

Prenatal alcohol exposure, especially during early pregnancy, can lead to fetal alcohol syndrome. The pharmacological and toxicological mechanisms of ethanol are related to the effects of ceramide. In this study, we established an alcohol exposure model in wild-type mice and in knockout mice for the key enzyme involved in ceramide metabolism, sphingomyelin synthase 2. This model received daily intragastric administration of 25% ethanol, and pups were used at postnatal days 0, 7, 14, 30 for experiments. Serology and immunofluorescence staining found that ethanol exposure dose-dependently reduced blood sphingomyelin levels in two genotypes of pups, and increased neural cell proliferation and the number of new neurons in the hippocampal dentate gyrus. Western blot analysis showed that the relative expression level of protein kinase C α increased in two notypes of pups after ethanol exposure. Compared with wild-type pups, the expression level of the important activator protein of the ceramide/ceramide-1-phosphate pathway, protein kinase C α, was reduced in the hippocampus of sphingomyelin synthase 2 knockouts. Our findings illustrate that ceramide is involved in alcohol-induced neural proliferation in the hippocampal dentate gyrus of pups after prenatal ethanol exposure, and the mechanism may be associated with increased pression of protein kinase C α activating the ceramide/ceramide-1-phosphate pathway.

Key Words

neural regeneration; brain injury; ceramide; neural cells; proliferation; prenatal alcohol exposure; sphingomyelin synthase 2 knockout mice; sphingomyelin; sphingomyelin synthase; ceramide-1-phosphate; fetal alcohol syndrome; grants-supported paper; neuroregeneration

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Author contributions: Wang ZX, Deng TX, Deng JX carried out the detailed experiments. Gao XQ was involved in data analysis and carried out all the statistical tests. Shi YY and Ma ZY were responsible for the prenatal alcohol exposure model and carried out the enzymatic assays. Liu B and Jin HX measured the blood alcohol concentration and sphingomyelin levels of different treatment groups. Deng JB conceived the experiment and was responsible for overall project supervision. Deng JB and Deng JX were involved in writing the paper. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

Ethical approval: The study was approved by the Henan Branch of the Chinese Medical Association Animal Ethics Committee in China.

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INTRODUCTION

Acute alcohol exposure can cause a large amount of nerve cell apoptosis, but can it cause nerve cell proliferation? Which mechanisms are involved in the regulation of this phenomenon? This paper focuses on nerve cell proliferation in the hippocampal dentate gyrus, because this region belongs to the ancient cortex and it is the storage area for memories and learning. The hippocampus is also a relatively active area of nerve cell proliferation[1].

Long-term excessive consumption of alcohol can cause damage to multiple organs and systems of the body, including the digestive, cardiovascular, immune, and nervous systems[2]. Alcohol can cross through the blood-brain barrier and directly cause neurotoxicity to the central nervous system, leading to the apoptosis of nerve cells and a decrease in synapses[3]. Brain functions can even be altered, with the development of cognitive impairments and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's diseases[4-6]. Clinical literature and animal experiments have shown that prenatal alcohol exposure *in utero*, especially during the early stages of pregnancy, can cause stillbirth, miscarriage, premature birth, and even fetal alcohol syndrome[6-7]. Children with fetal alcohol syndrome may have body deformities, abnormalities in organ development, mental retardation, and functional impairments in the central nervous system[6].

The mechanisms underlying alcohol neurotoxicology are not yet clear. Some scholars believe that alcohol causes its effects by inhibiting NMDA receptors[8-10] and activating GABA receptors[11-13]. Alcohol can also induce cellular apoptosis by influencing the activity of Na^+-K^+-ATPase and adenylate cyclase, as well as by modulating the opening of Ca^{2+} channels[14]. Other scholars believe that the pharmacological and toxicological mechanisms involve ceramide[15-16]. As an important signal transduction molecule, ceramide participates in a variety of cellular transduction pathways and can modulate cell cycle, cellular differentiation, proliferation, and apoptosis (including autophagy). Ceramide also participates in many cellular physiological functions, such as stress, immune response and inflammation[17]. Ceramide is an intermediate-type sphingolipid, whose phosphorylation, glycosylation and acylation products participate in different cellular physiological functions[18]. The main metabolic product of ceramide is to transform into sphingomyelin through sphingomyelin synthase, which plays a central role in the metabolic process of sphingolipids and phospholipids. This enzyme also participates in the modulation of cellular ceramide and diacylglycerol levels[19-21]. In the mammalian genome, sphingomyelin synthase (SMS) has two isozymes, SMS_1 and SMS_2. The loss of SMS_1 or SMS_2 genes through RNA interference would decrease the synthesis of sphingomyelin, resulting in the accumulation of ceramide and a subsequent increase in its cellular effects[22]. In this project, we constructed a prenatal alcohol exposure model using SMS_2 knockout ($SMS_2^{-/-}$) mice to investigate the role of ceramide in the process of alcohol-induced nerve cell proliferation.

Our study has the following characteristics: (1) We used $SMS_2^{-/-}$ mice to establish an alcohol exposure model. (2) Experimental design and subgroup analyzes were random. (3) The analysis aimed to elucidate the relationships among alcohol exposure, ceramide, and neural proliferation.

Increasing evidence has confirmed that alcohol can induce nerve cell apoptosis, but few studies have observed whether alcohol can promote nerve cell proliferation. Our research is the first in China to establish an alcohol exposure model in gene knockout mice. Our aims in this study are: to determine if alcohol can induce nerve cell proliferation, and the mechanisms underlying this process; to investigate whether SMS_2 gene knockout affects alcohol-induced neural proliferation, and to clarify the role of ceramide and pathways involved in the above process.

RESULTS

Quantitative analysis of experimental animals

Thirty wild-type female mice and thirty $SMS_2^{-/-}$ mice (pregnant) were randomly divided into three groups: low-dose alcohol group (intragastric administration of 25% alcohol 2.0 g/kg per day), high-dose alcohol group (intragastric administration of 25% alcohol 4.0 g/kg per day), and control group (intragastric administration of distilled water per day). Fifteen pups were selected at postnatal days 0, 7, 14, 30 (P0, P7, P14, P30). A total of 360 pups were used for experiments. Among them, ten pups in each group were applied for immunohistochemical staining while 5 pups for western blot assay. The present study is an *in vivo* experiment and the pups were processed immediately after selection. There was no loss of animals.

Effect of prenatal alcohol exposure on serum sphingomyelin levels

The $SMS_2^{-/-}$ mice could not transform ceramide into sphingomyelin due to the deletion of the SMS_2 gene; therefore, ceramide accumulated in the body, including central nervous system and blood. Since sphingomyelin level was inversely related to the concentration of ceramide, serum sphingomyelin levels could roughly reflect the level of ceramide accumulation in the animals. In our study, the serum sphingomyelin levels were measured in P0 pups, and the comparisons were made between $SMS_2^{+/+}$ and $SMS_2^{-/-}$ pups (Figure 1). The serum sphingomyelin level of $SMS_2^{-/-}$ pups were significantly lower than that of wild-type pups ($P < 0.01$). Prenatal alcohol exposure could decrease the sphingomyelin levels in both $SMS_2^{+/+}$ and $SMS_2^{-/-}$ pups with in a dose dependent manner ($P < 0.05$). This evidence suggests that alcohol exposure could decrease the activity of SMS_2, resulting in the large accumulation of ceramide in cells following the suppression of the ceramide pathway.

Effect of prenatal alcohol exposure on neural proliferation in the hippocampal dentate gyrus

Normally, there are stem cell pools in the central nervous system even in adulthood, for example in the subgranular zone of the dentate gyrus, and these neural stem cells have long-term proliferative capabilities[23]. Understanding the normal development of neural proliferation in the dentate gyrus would be the prerequisite for understanding the effects of alcohol on neural proliferation. First, we observed proliferative cells in the dentate gyrus of wild-type mice at P0, P7, P14, and P30. The general pattern was that neural proliferation decreased with increasing age. At P0, the proliferative cells in wild-type pups were distributed widely throughout the hippocampus and dentate gyrus. At P7, the proliferative cells were mainly located in the dentate gyrus, including the hilus, subgranular zone, granular layer and molecular layer. At P14, the proliferative cells were located in the subgranular zone and granular layer. However, at P30 (adulthood), the proliferative cells were mainly limited to the subgranular zone, suggesting that neural proliferation in the dentate gyrus of wild-type pups gradually decreased with as age increased (Figure 2). This principle of neural proliferation also was demonstrated in $SMS_2^{-/-}$ mice (Figure 3). Statistical analysis (Figure 4) indicated that neural proliferation in the dentate gyrus gradually decreased with increasing age in wild-type ($F = 483.84$, $P < 0.001$) or $SMS_2^{-/-}$ pups ($F = 873.92$, $P < 0.001$).

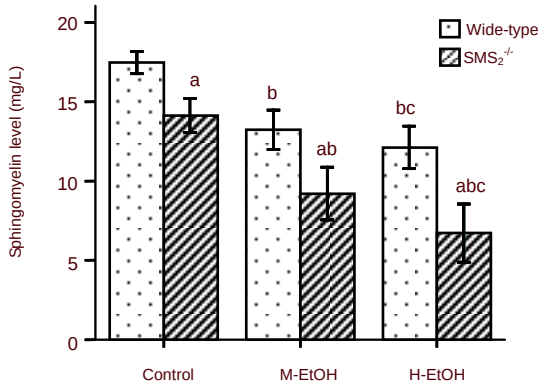

Figure 1 Effect of prenatal alcohol exposure on serum sphingomyelin levels in pups at postnatal day 0.

The serum sphingomyelin levels in $SMS_2^{-/-}$ pups were significantly lower than wild-type pups. Serum sphingomyelin level decreased with increasing ethanol dose in the two groups of pups. $^aP < 0.01$, *vs*. wild-type group; $^bP < 0.01$, *vs*. control group (from the same genome); $^cP < 0.05$, *vs*. M-EtOH group (from the same genome). Sphingomyelin levels are shown as mean ± SD in 15 pups for each group. One-way analysis of variance and Student-Newman-Keuls test were used for statistical analysis.

M-EtOH: Moderate-dose ethyl alcohol treatment (2.0 g/kg per day); H-EtOH: high-dose ethyl alcohol treatment (4.0 g/kg per day); $SMS_2^{-/-}$: sphingomyelin synthase 2 knockout.

Prenatal alcohol exposure could significantly increase neural proliferation in the dentate gyrus in both the wild-type (Figure 2) and $SMS_2^{-/-}$ pups (Figure 3) in a dose-dependent manner (Figures 2, 3). Statistical analysis further confirmed our observation (Figure 4). Neural proliferation in the dentate gyrus of alcohol-treated groups was significantly higher than that of the control group ($P < 0.01$), with the high-dose alcohol group being significantly more than the low-dose group ($P < 0.05$).

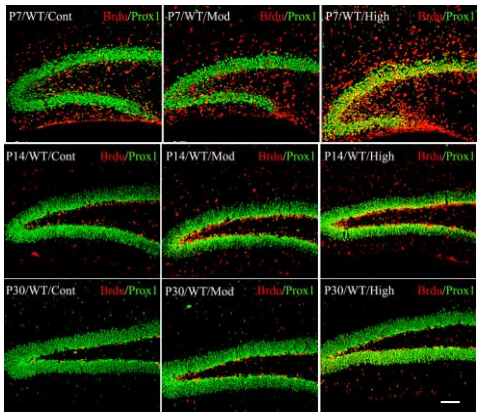

Figure 2 Effect of prenatal alcohol exposure on neural proliferation in the hippocampal dentate gyrus of wild-type pups at postnatal days 7, 14, 30 (laser confocal microscope, × 20).

The granular cells were immunocytochemically labeled with Prox 1 (green), and proliferative nerve cells were labeled with BrdU (red). Prenatal alcohol exposure can induce neural proliferation in a dose-dependent manner.

WT: Wild-type group; Cont: control group; Mod: moderate-dose alcohol (2.0 g/kg per day) group; High: high-dose alcohol (4.0 g/kg per day) group. Scale bar: 80 μm.

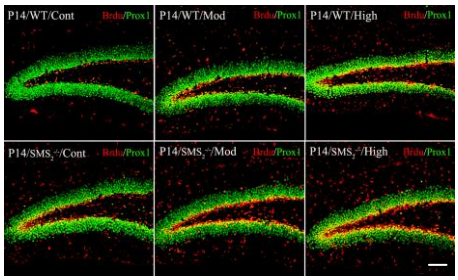

Figure 3 Neural proliferation in wild-type (WT) *versus* sphingomyelin synthase 2 knockout ($SMS_2^{-/-}$) pups at postnatal day 14 (laser confocal microscope).

Alcohol exposure could upregulate neural proliferation in the dentate gyrus of both WT and $SMS_2^{-/-}$ pups in a dose-dependent manner. For both the control and alcohol groups, neural proliferation in the dentate gyrus of $SMS_2^{-/-}$ pups was significantly higher than that of the WT pups. The granular cells (green) and proliferative nerve cells (red) were labeled using immunocytochemistry.

WT: Wild-type group; Cont: control group; Mod: moderate-dose alcohol (2.0 g/kg per day) group; High: high-dose alcohol (4.0 g/kg per day) group. Scale bar: 80 μm.

The same pattern was observed in $SMS_2^{-/-}$ pups ($P < 0.05$), suggesting that prenatal alcohol exposure could dose-dependently induce neural proliferation in a long-lasting manner. To understand the roles of ceramide in alcohol-induced neural proliferation, neural proliferation was compared in wild-type and $SMS_2^{-/-}$ pups with and without alcohol exposure. Our findings showed that neural proliferation in the dentate gyrus of $SMS_2^{-/-}$ pups was greater than that of the wild-type pups (Figure 3). Statistical analysis (Figure 4) also confirmed that the level of neural proliferation in the dentate gyrus of $SMS_2^{-/-}$ knockouts was higher than that of the wild-types ($P < 0.05$), suggesting that the accumulation of ceramide upregulated alcohol-induced neural proliferation.

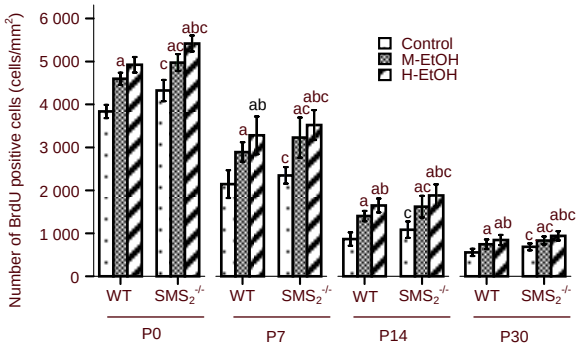

Figure 4 Effect of prenatal alcohol exposure on neural proliferation in the dentate gyrus from pups of differing ages.

BrdU positive cells are quantified in ten pups for each group (data were expressed as mean ± SD). One-way analysis of variance followed by Student-Newman-Keuls test and least significant difference test was performed. Neural proliferation in the dentate gyrus gradually decreased with increasing age in both wild-type or $SMS_2^{-/-}$ pups. Ethanol could increase neural proliferation (in a long-lasting manner) in a dose-dependent manner. Moreover, neural proliferation in the $SMS2^{-/-}$ pups was higher than that of the WT pups. $^aP < 0.01$, *vs*. control group (from the the same genome); $^bP < 0.05$, *vs*. M-EtOH group (from the same genome); $^cP < 0.05$ *vs*. WT group.

WT: Wild-type; $SMS_2^{-/-}$: sphingomyelin synthase 2 knockout; M-EtOH: moderate-dose ethanol (2.0 g/kg per day); H-EtOH: high-dose ethanol (4.0 g/kg per day).

Effect of prenatal alcohol exposure on newborn neurons in the dentate gyrus of wild-type and SMS_2 knockouts

To further demonstrate whether ceramide is involved in ethanol-induced neural proliferation in the dentate gyrus, newborn neurons were labeled in the different treatment groups. Doublecortin is a microtubule-associated protein used to label migrating and differentiating newborn neurons[24]. In mammals, doublecortin positive neurons were distributed in a similar pattern to that of the proliferative cells in the dentate gyrus[24]. In this study, the newborn neurons in the dentate gyrus were specifically labeled with doublecortin to further observe whether alcohol-induced ceramide accumulation can influence the formation of newborn neurons. In our experiments, the development and distribution pattern of newborn neurons were similar to those of the proliferative neural cells. Mainly distributed in the subgranular zone of the dentate gyrus, newborn neurons presented with the morpholog-

ical characters of granular cells (Figure 5). Although the number of newborn neurons in the dentate gyrus decreased with age increase, there was a quantitative difference between SMS_2 knockouts and wild-types (P < 0.05). At the same age, the SMS_2 knockout pups displayed more newborn neurons than wild-type pups.

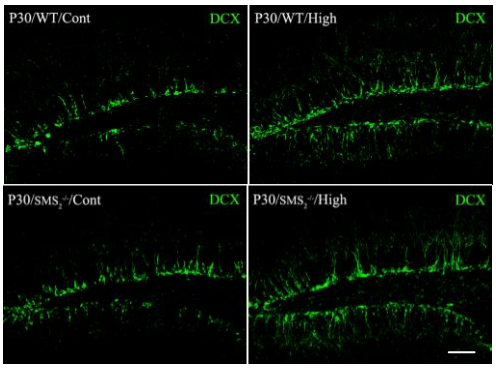

Figure 5 Effect of prenatal alcohol exposure on newborn neurons in the hippocampal dentate gyrus of pups at postnatal day 30 (laser confocal microscope).

Doublecortin (DCX, green) positive newborn neurons were labeled using immunocytochemistry. The number of newborn neurons in the dentate gyrus of $SMS_2^{-/-}$ pups was much higher than that of the wild-type pups, and alcohol could upregulate the formation of newborn neurons.

WT: Wild-type; $SMS_2^{-/-}$: sphingomyelin synthase 2 knockout; Cont: control group; High: high-dose alcohol group. Scale bar: 80 μm.

Moreover, prenatal alcohol exposure could induce the upregulation of newborn neurons in both the wild-type and SMS_2 knockout pups, and the newborn neurons in the high-dose alcohol group were densely concentrated with long extensions (Figure 5). The number of newborn neurons in both wild-type and SMS_2 knockout pups after prenatal alcohol exposure was significantly higher than that of the control groups (P < 0.01). This increase occurred in a dose-dependent manner (P < 0.05; Figure 6), suggesting that prenatal alcohol exposure could induce the formation of newborn neurons and that ceramide played an important role in this process.

Role of protein kinase C α in the ceramide 1-phosphate signal pathway

Protein kinase C α is a key protein and an important marker for the evaluation of the activity of the ceramide pathway. Inhibition of protein kinase C α activity could block the ceramide pathway and ceramide-1-phosphate-induced cell mitosis[25]. In our study, protein kinase C α-positive cells in the hippocampus were visualized with immunocytochemistry. Protein kinase C α was mainly expressed in the cytoplasm of cells in the neocortex, subventricular zone, hippocampus, dentate gyrus, hilus and brain stem, suggesting that protein kinase C α may participate in the majority of signal transduction processes in nerve cells.

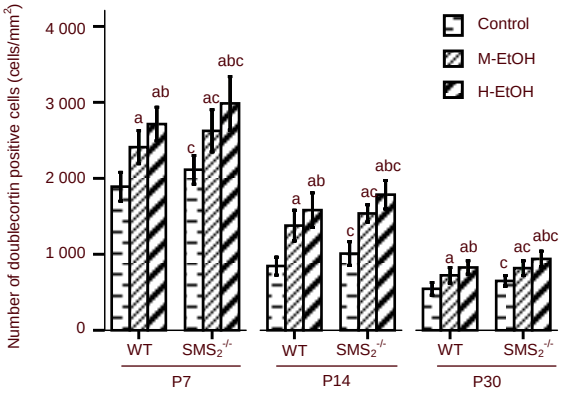

Figure 6 Effect of prenatal alcohol exposure on newborn neurons in the hippocampal dentate gyrus from pups of differing ages.

The number of newborn neurons in the dentate gyrus of $SMS_2^{-/-}$ pups was much higher than that of the WT pups. Alcohol could upregulate the formation of newborn neurons. The number of doublecortin positive cells is shown as mean ± SD in ten pups for each group. One-way analysis of variance followed by Student- Newman-Keuls test and least significant difference test was performed. ^aP < 0.01, *vs*. control group (from the same genome); ^bP < 0.05, *vs*. M-EtOH group (from the same genome); ^cP < 0.05, *vs*. WT group.

WT: Wild-type; $SMS2^{-/-}$: sphingomyelin synthase 2 knockout; M-EtOH: moderate-dose alcohol (2.0 g/kg per day); H-EtOH: high-dose alcohol (4.0 g/kg per day).

As shown in Figure 7, protein kinase C α (green) was expressed in either NeuN (red) positive mature neurons or newborn neurons, which were mainly located in the subgranular layer. To prove the effects of protein kinase C α expression on alcohol-induced neural proliferation, we used western blot assay to semi-quantitatively detect the relative expression level of protein kinase C α in the hippocampus of different groups at P7 (Figure 8A). Compared with wild-type pups, the expression of protein kinase C α in the hippocampal cells of SMS_2 knockouts was relatively low. After alcohol exposure, protein kinase C α expression in the two genotypic groups increased. In other words, the level of protein kinase C α expression in the alcohol-treated groups was significantly more than that of the control group. This expression increase occurred in a dose-dependent manner. Semi-quantitative analysis results (Figure 8B) indicated that the relative expression level of protein kinase C α in the hippocampal cells of wild-type pups was 0.32 ± 0.03, but it was decreased to 0.24 ± 0.02 in SMS_2 knockouts (P < 0.05). After prenatal alcohol exposure, the relative protein ki-

nase C α expressions in two genotypic groups increased ($P < 0.01$) in a dose- dependent manner.

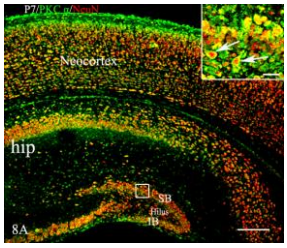

Figure 7 Effect of prenatal alcohol exposure on the expression of protein kinase C (PKC) α in wild-type pups at postnatal day 7.

Protein kinase C α was expressed in the cytoplasm of nerve cells in the neocortex, subventricular zone, hippocampus (hip), dentate gyrus and the hilus. Inset in the right upper corner shows a high magnification image of the suprapyramidal blade (SB) of the granular layer. Protein kinase C α could be expressed in both neural stem cells of the subgranular layer, and granular cells co-expressing neuronal nuclei NeuN; arrows). The infrapyramidal blade (IB) of the granular layer is labeled as well. Scale bar: 200 μm.

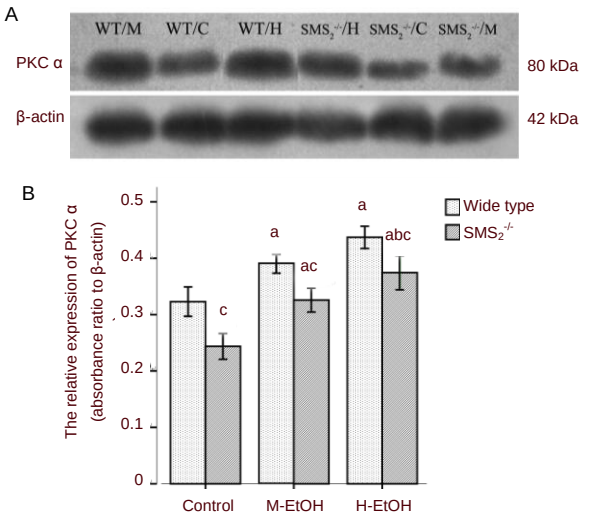

Figure 8 Expression of protein kinase C (PKC) α in the hippocampus of different genotypic pups at postnatal day 7.

(A) Western blot analysis of the relative expression of PKC α in the hippocampus of pups. (B) Semi-quantitative western blot analysis of PKC α expression in the hippocampus of pups. PKC α expression levels are expressed as mean ± SD in five pups per group. One-way analysis of variance followed by Student-Newman-Keuls test was performed. $^{a}P < 0.01$, *vs*. control group; $^{b}P < 0.05$, *vs*. M-EtOH group; $^{c}P < 0.05$, *vs*. WT group.

WT: Wild-type; $SMS_2^{-/-}$: sphingomyelin synthase 2 knockout; WT/C: control group of WT; WT/M: WT moderate-dose alcohol group; WT/H: WT high-dose group; $SMS_2^{-/-}$/C: control group of $SMS_2^{-/-}$; $SMS_2^{-/-}$/M: moderate-dose alcohol group of $SMS_2^{-/-}$; $SMS_2^{-/-}$/H: high-dose group of $SMS_2^{-/-}$; M-EtOH: moderate-dose alcohol treatment (2.0 g/kg per day); H-EtOH: high-dose alcohol (4.0 g/kg per day).

DISCUSSION

As an effective toxin to the developing nervous system, prenatal alcohol exposure *in utero* can cause wide-ranging neuropathological damage, and even fetal alcohol syndrome, which seriously affects the development of multiple organ systems in the fetus, especially the central nervous system[26-27]. The influences of alcohol on the central nervous system mainly occur through certain neurotransmitter receptors[28], such as the glutamate (GluR2/3, NMDAR), γ-aminobutyric acid, neural acetylcholine[29], and the dopamine receptors[30]. Alcohol can also activate downstream signal pathways, such as the c-Jun N-terminal kinase pathway[31] to induce cell apoptosis[32].

Recently, Saito *et al* [33] have found that alcohol could induce apoptosis of nerve cells through the *de novo* synthesis of ceramide, eventually leading to pathological neurodegeneration and compensatory neural proliferation. In the ceramide pathway, ceramide-1-phosphate (produced through the phosphorylation of ceramide by ceramide kinase) and extracellular signal-regulated protein kinase are key elements to induce cell apoptosis and compensatory proliferation[25]. But it is not yet certain whether alcohol-induced neural proliferation was specifically achieved through the ceramide pathway. In this study we constructed a prenatal alcohol exposure model using SMS_2 knockout pups and sought to prove our hypothesis that alcohol-induced ceramide accumulation would upregulate neural proliferation in the hippocampus.

Decreased blood sphingomyelin levels in both wild-type and SMS_2 knockout pups following prenatal alcohol exposure

Since the SMS_2 gene was deleted, ceramide could not be metabolized into sphingomyelin smoothly and would subsequently accumulate in cells, tissues and blood[34]. However, the involvement of ceramide in ethanol toxicity is not clear. To study this involvement, blood sphingomyelin level which indirectly reflects the level of ceramide was measured in different alcohol treatment groups of both SMS_2 knockout and wild-type pups at P0. Our findings showed that blood sphingomyelin level of SMS_2 knockouts was significantly lower than that of wild-type pups, and alcohol could reduce blood sphingomyelin level in a dose-dependent manner. Generally, the level of sphingomyelin is closely related to ceramide metabolism, because the synthesis of sphingomyelin requires the participation of SMS. In mammals, SMS is present as two isozymes: SMS_1 (mainly concentrated in golgi bodies) and

SMS_2 (mainly distributed to cell membrane)[35-36]. Ceramide is mainly transported extracellularly for SMS_2-catalyzed synthesis of sphingomyelin. There is evidence that the loss or inactivation of SMS_2 can block the synthesis of sphingomyelin, causing a decrease sphingomyelin level in blood and the accumulation of intracellular ceramide, which will cause different cellular effects through downstream signaling cascade reactions[22, 37]. The alcohol-induced decrease of sphingomyelin levels suggested that ceramide was involved in the mechanisms of alcohol toxicity.

Neural proliferation caused by alcohol-induced ceramide accumulation

The regulatory mechanisms of neural proliferation during development are not understood well. Signal pathways thought to be involved in neural proliferation include MAPK[38], Wnt[39], tumor growth factor-β[40] and Shh[41]. Recently, the ceramide-1-phosphate pathway was shown to be involved in the upregulation of neural proliferation[42-43]. In this study, we wanted to know specifically whether the ceramide/ceramide-1-phosphate pathway participated in the upregulation of alcohol-induced neural proliferation. SMS_2 knockout mice served as a good model to study the relationships between alcohol-induced neural proliferation and ceramide modulation. During adulthood, only some areas in the central nervous system, such as the subventricular zone, the subgranular layer of the dentate gyrus and the rostral migratory stream in the olfactory bulb retain the capacity of cell proliferation[44]. In the present study, the dentate gyrus was used as a target to study the alterations in neural proliferation after alcohol exposure. Our studies indicated that alcohol could induce compensatory neural proliferation and the formation of newborn neurons in a long-term, dose dependent manner. We found that neural proliferation of the alcohol-treated groups, for both the wild-type and SMS_2 knockouts, were higher than the control groups. Neural proliferation and the number of newborn neurons increased in a dose-dependent manner with higher proliferation resulting from higher dosage of prenatal alcohol exposure. Neural proliferation was probably the result of a compensatory mechanism for neuroapoptosis induced by alcohol[45]. The proliferative stem cells responded to this apoptosis with mitotic divisions and modulations of the cell cycle, thus causing compensatory neural proliferation. The differentiated polymorphic neurons would migrate into the hippocampus or the cortex, to replace some apoptotic cells and maintain functional completeness of the nervous system[32, 46]. To further understand how alcohol-induced ceramide accumulation upregulated neural proliferation and to explore the neurotoxicological functions and mechanisms of alcohol toxicity, neural proliferation in SMS_2 knockout pups after prenatal alcohol exposure was observed. In our experiments, neural proliferation in the dentate gyrus of SMS_2 knockout pups was significantly higher than the wild-type pups at the same age, resulting in more newborn neurons. This is probably due to the accumulation of intracellular ceramide following the loss of the SMS_2 gene leading to subsequent compensatory cell proliferation. As one type of biologically active sphingolipid, ceramide is important for signal transduction functions in most cells. The ceramide/ceramide-1-phosphate pathway mainly enhanced cell mitosis and cell migration[47-48] through the activation of phosphatidylinositol 3 kinase/ protein kinase B and protein kinase C α signaling[49], which is most likely the case in our experiments.

Activation of protein kinase C α in alcohol-induced neural proliferation

Ceramide is a bioactive sphingolipid that regulates critical biological functions, including apoptosis and cell proliferation[50-51]. Neural proliferation is mainly achieved through its phosphorylated metabolite, ceramide-1-phosphate. In this process, protein kinase C α plays an important role in the activity of ceramide/ceramide-1-phosphate signaling[52]. First, the phosphorylation of protein kinase C α needs to be activated by SMS, and then the phosphorylated protein kinase C α can subsequently enhance the activation of phosphatidylinositol 3-kinase/protein kinase B for the downstream modulation of ceramide-1-phosphate activity directly. Eventually, ceramide-1-phosphate would cause the influx of Ca^{2+} into cells to stimulate cell apoptosis and proliferation[25, 53-54]. Therefore, a major factor leading to the stimulation of cell proliferation is the activation of protein kinase C α isoforms, whose activation requires their recruitment to the cell membranes[55]. Ceramide-1-phosphate was able to induce the phosphorylation of this important site of protein kinase C α. In this way, measuring protein kinase C α expression would be helpful to understand how ceramide regulates alcohol-induced neural proliferation. Immunocytochemistry showed that protein kinase C α was expressed in most nerve cells, especially in proliferative cells, suggesting that protein kinase C α is necessary for neuronal activity and neural proliferation. The semi-quantitative analysis indicated that the expression level of protein kinase C α in the hippocampal neurons of SMS_2 knockout pups was relatively low. This is because the lack of SMS in SMS_2 knockout pups could decrease the activity of protein kinase C α, thereby slowing the ceramide/ceramide-1-phosphate cascade process[25]. By contrast, alco-

hol could induce the expression of protein kinase C α in a dose-dependent manner. This suggests that alcohol-induced accumulation of ceramide could upregulate neural proliferation, and that protein kinase C α phosphorylation is a key step for the activation of the ceramide pathway.

In summary, serum sphingomyelin level in SMS_2 knockout pups was significantly lower than that of wild-types. Alcohol exposure could decrease sphingomyelin levels in both wild-type and SMS_2 knockouts in a dose-dependent manner. Interestingly, SMS_2 knockouts revealed a greater amount of neural proliferation in the dentate gyrus compared with wild-type pups, and alcohol could further induce neural proliferation in a long-term and dose-dependent manner. Protein kinase C α phosphorylation was probably a key step for the activation of the ceramide pathway. Alcohol-induced expression of protein kinase C α could activate the ceramide-1-phosphate signaling cascade to enhance neural proliferation and the formation of newborn neurons.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

The experiment was performed at the Institute of Neurobiology, College of Life Science, Henan University, China, between March 2009 and March 2011.

Materials

SMS_2 heterozygous mice (one male and two female, average weight 21.2 ± 1.7 g, clean level, animal license number: NSJXC-79J14537) were donated by Professor Xiancheng Jiang from the State University of New York Downstate Medical Center, USA. Ten male and twenty female wild-type (C57BL/6J) mice (average weight 20.7 ± 2.1 g, clean level, animal license No. HNKF36790131) were provided by the Experimental Animal Center of Henan University, China. A total of 360 pups from P0–P30 were used. Offspring were produced from the heterozygotes, and their genotypes were identified by PCR assay. Primer information is listed in Table 1. SMS_2 genotype identification was then performed as previously described[56]. Two possible bands could be identified at 250 and 145 (bp) on the gel image. The wild-type sample had only the 145 (bp) band, while the heterozygous sample had both 250- and 145-bp bands. The knockout sample had only the 250 bp band. All experiments were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[57], to ensure animal welfare during experiments.

Table 1 The information about primers

Primer	Sequence
Upstream primer 1	5′-GTG GCG GAC AAT GGA TAT CAT AGA GAC AGC-3′
Upstream primer 2	5′-GCC AGA GGC CAC TTG TGT AGC-3′
Downstream primer	5′-CAT AAG GTC TTG GGT TTG CCC TTG CC-3′

Methods

Establishment of the alcohol exposure model

SMS_2 knockout females were checked each morning for the presence of a vaginal plug; a positive plug was defined as E0. Embryonic (E = day of conception, E0 = vaginal plug found in mated females) or postnatal offspring (P = postnatal days, P0 = the first 24 hours after birth) were produced from timed pregnancies. Postnatal pups were genotyped using PCR, and the wild-type and SMS_2 knockout pups were assigned to one of two groups. (1) Alcohol treatment groups: females received a daily intragastric gavage of 25% (w/v) alcohol [Kaifeng Civilized (Group) Co., Ltd., Henan Province, China] at a dose of either 2.0 g/kg or 4.0 g/kg, which began at E5 and continued to parturition. To allow the stomach to empty and to facilitate the absorption of alcohol, food access was removed 2 hours before alcohol dosing. Animals were weighed and gavaged at the same time each day. (2) Control groups: chow fed females were allowed free access to food and water except in the morning when food was removed for 2 hours prior to weighing. The pair fed females were intubated with the same quantity of isocaloric and isovolumetric maltose-dextrin as the alcohol treatment groups. Because there were no significant differences in neural proliferation between the pair fed and chow fed groups, the data from the two control groups were combined to increase the statistical power of the analysis[9]. To understand the long-term effect of ethanol toxicity on the development of the central nervous system, pups were harvested at P0, P7, P14 and P30. At least 15 pups were used in each treatment group at P0, P7, P14, and P30, with a total of 230 pups used. Ten pups of each group were used for immunohistochemistry, and five pups were used for western blot analysis.

Measurements of blood alcohol concentration

On E10 and E15, the tail tips of pregnant mice treated with alcohol were nicked with scissors at 60 and

120 minutes after gavage, and 10 μL of blood was collected into heparinized hematocrit tubes. Approximately 5 μL serum was prepared by centrifugation and blood alcohol concentration was determined using an ANALOX model GL5 Analyser (Germany). Four blood alcohol concentration values were obtained from each dam (two readings on E10 and E15, respectively). The highest of the four values was defined as the peak blood alcohol concentration value of the dam and her offspring. The peak blood alcohol concentration value of 4.0 g/kg group was in the range of 30–50 mg/L, while that of 2 g/kg group was in the range of 15–30 mg/L. These groups were accordingly designated as the high and moderate alcohol treatment groups.

Measurements of serum sphingomyelin levels

P0 pups of each group were obtained, and blood was collected through cardiac puncture. The sphingomyelin level was analyzed using enzymatic assays. Briefly, the SMase hydrolyzed sphingomyelin was added into the phosphorylcholine and n-acylsphingosine, then, alkaline phosphatase generated choline from phosphorylcholine. Choline could then be used to generate hydrogen peroxide in a reaction catalyzed by choline oxidase. Finally hydrogen peroxide was used together with DAOS, 4-aminoantipyrine, and peroxidase, as a catalyst, to generate a blue dye with an optimal absorption at 595 nm. The concentration of blue dye, which reflected the concentration of sphingomyelin, was analyzed using a Bio-Rad 680 automatic microplate reader (Bio-Rad, CA, USA) at 595 nm. The sphingomyelin concentration in blood could be calculated based on the standard curve[58].

Immunohistochemistry

Pups at various ages (P0, P7, P14, P30) were anesthetized with sodium pentobarbital (20 mg/kg) and perfused transcardially with 4% paraformaldehyde. Brains were removed, and immersion-fixation was continued at 4°C for 1–2 days. Coronal brain slices (50 μm) were then sectioned using a vibratome. After washing three times with 0.1 mol/L PBS, slices were treated with different primary antibodies and the corresponding secondary antibodies for immunofluorescent single- or double-labeling. The primary antibodies include rabbit anti-doublecortin (DCX) polyclonal antibody (1:200; #4604, Cell Signaling, Danver, MA, USA), rabbit anti-Prox 1 polyclonal antibody (1:400; AB5475, Chemicon, Billerica, MA, USA), rabbit anti-protein kinase C α polyclonal antibody (1:50; P4334, Sigma, St. Louis, MO, USA), and mouse monoclonal anti-NeuN antibody (1:500; MAB377B, Millipore, Billerica, MA, USA). After incubating at 4°C overnight, slices were washed three times with 0.1 mol/L PBS and then treated with secondary antibodies. The corresponding secondary antibodies were Alexa Fluor 568 donkey anti-mouse IgG (1:300; A10037, Invitrogen Life Technologies, Carlsbad, CA, USA) and Alexa Fluor 488 donkey anti-rabbit IgG (1:600; Invitrogen, A21206). After incubating with secondary antibody at room temperature for 3 hours, slices were washed three times with 0.1 mol/L PBS and were subsequently mounted with fluorescent mounting medium. With the excitation of rhodamine and FITC (Jianglaibio, Shanghai, China), the slices were observed and photographed under a fluorescent microscope (BX61, Olympus, Tokyo, Japan). Well labeled slices were then observed and imaged under laser scanning confocal microscopy (Olympus).

BrdU immunofluorescent labeling

BrdU (Sigma, B-5002) dissolved at sterile 7 mol/L in NaOH (7.5 mg/500 μL) in physiological saline was injected at a dose of 5 μg/mg into pregnant mice at E18 or into pups at P6, P13 and P29. To intensify labeling, a second identical dose was given 3 hours later. Animals were sacrificed 1 day after injection (at P0, P7, P14 and P30 respectively). The postnatal pups were transcardially perfused and brains were post-fixed in 4% paraformaldehyde in 0.1 mol/L PBS overnight at 4°C. After fixation, coronal brain sections (50 μm) were cut using a vibratome. To visualize nuclear BrdU incorporation, sections were incubated in 2 mol/L HCl at 37°C for 30 minutes to denature the DNA. Sections were then thoroughly rinsed with boric acid (pH 8.0) three times (10 minutes each time) and then with 0.1 mol/L PBS three times (10 minutes each time) to fully remove HCl within the slices. To visualize the neural proliferative cells and granular cells in the dentate gyrus, rabbit anti-Prox 1 polyclonal antibody (1:400; Chemicon, AB5475) and mouse anti-BrdU monoclonal antibody (1:100; ZSGB-BIO, Beijing, China, ZM-0013) were used for immunofluorescent double-labeling. The slices were incubated with all primary antibodies at 4°C overnight. After washing three times with 0.1 mol/L PBS, slices were incubated with secondary antibodies for 3 hours at room temperature. The secondary antibodies were Alexa Fluor 568 donkey anti-mouse IgG (1:300; Invitrogen, A10037) and Alexa Fluor 488 donkey anti-rabbit IgG (1:600; Invitrogen, A21206). After rinsing and mounting, the laser confocal microscope (Olympus, FV10) was used to observe and photograph Prox 1 and BrdU positive cells.

Western blot analysis of protein kinase C α expression in the hippocampi

Because ceramide possibly affects neural proliferation through ceramide-1-phosphate and protein kinase C α, we analyzed the level of protein kinase C α of the ceramide-1-phosphate loop using western blot analysis. P7 pups of each group were collected and sacrificed by cervical dislocation. After opening the skull, brains were quickly dissected away. The hippocampi were separated for homogenization. The cytoplasmic proteins of hippocampus were extracted using a protein extraction kit (Shanghai Beyotime Institute of Biotechnology, Shanghai, China, P0027). The collected cytoplasmic proteins were quantified using the Coomassie brilliant blue G250 method[59]. After standard curves were created, the sample protein concentration in different treatment groups was measured. Then, the proteins were electrophorized and subsequently electro-transfer red onto membranes. Rabbit anti protein kinase C α polyclonal antibody (1:20 000; Sigma, P4334) was then added to the membrane and incubated at 4°C overnight. Horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (1:1 000; Beijing Solarbio Science & Technology) was then added to the membrane for a 2-hour incubation at room temperature. Prior to each incubation, PBS washing was performed three times, for 5 minutes each. After the enhanced chemiluminescence reagent (ZSGB-BIO, Beijing) reaction for 2 minutes, the protein bands were exposed to film for subsequent development and analysis. β-actin, detected with mouse anti monoclonal antibody (1:1 000; Shanghai Beyotime Institute of Biotechnology, Shanghai, China, #AA128), was used as the internal control. The absorbance ratio of positive bands to internal standard (β-actin) bands represented the relative expression level of the protein of interest.

Data analysis

Therefore, we chose to observe the dentate gyrus as our target area[24]. The measurement area of the dentate gyrus was targeted to the suprapyramidal blade, the part of the granule cell layer that is close to hippocampal area CA1 and located above the pyramidal cell layer in CA3. The number of proliferated cells (BrdU positive cells) and newborn neurons (doublecortin positive cells) in suprapyramidal blade (cells/mm^2, 20 × magnification, from the sub-granular layer to the super-granular layer) was measured and analyzed statistically by ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

Data were represented as mean ± SD. SPSS 17.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. One-way analysis of variance followed by Student-Newman-Keuls test and/or least significant difference test was performed. $P < 0.05$ was accepted for an indication of statistically significant difference.

Research background: Extensive clinical data and animal experiments have demonstrated that prenatal alcohol exposure, especially early pregnancy, can cause stillbirth, miscarriage, premature birth, and even lead to fetal alcohol syndrome. The neurotoxicology mechanisms of alcohol are not very clear.

Research frontiers: Some scholars believe that alcohol can induce cellular apoptosis by influencing the activity of Na^+/K^+-ATPase and adenylate cyclase, as well as by modulating the opening of Ca^{2+} channels. The pharmacological and toxicological mechanisms of alcohol may, however, depend on ceramide.

Clinical significance: Our findings may provide evidence for the pathogenesis of fetal alcohol syndrome and can help further in the understanding of the physiological function and significance of ceramide in nerve cells.

Academic terminology: Fetal alcohol syndrome: Mothers consuming large amounts of alcohol during pregnancy cause growth and development disorders in the fetus and postnatally.

Peer review: This is the first study in China to use SMS_2 knockout mice to establish an alcohol exposure model. This model enriches the knowledge surrounding the toxicological mechanisms of alcohol, and further reveals a new strategy for the prevention and treatment of fetal alcohol syndrome.

REFERENCES

[1] O'Leary OF, Zandy S, Dinan TG, et al. Lithium augmentation of the effects of desipramine in a mouse model of treatment-resistant depression: a role for hippocampal cell proliferation. Neuroscience. 2013;228:36-46.

[2] Dumitrescu RG, Shields PG. The etiology of alcohol-induced breast cancer. Alcohol. 2005;35(3):213-225.

[3] Cui ZJ, Zhao KB, Wen SG, et al. Effects of alcohol exposure during pregnancy on dendritic spine and synapse of visual cortex in filial mice. Yao Xue Xue Bao. 2010; 45(7):833-839.

[4] Jaatinen P, Rintala J. Mechanisms of ethanol-induced degeneration in the developing, mature, and aging cerebellum. Cerebellum. 2008;7(3):332-347.

[5] Cai L, Bian M, Liu M, et al. Ethanol-induced neurodegeneration in NRSF/REST neuronal conditional knockout mice. Neuroscience. 2011;181:196-205.

[6] Riley EP, Mattson SN, Thomas JD. Fetal alcohol syndrome. Encyclopedia Neurosci. 2009;213-220.

[7] Chasnoff IJ, Wells AM, Telford E, et al. Neurodevelopmental functioning in children with FAS, pFAS, and ARND. J Dev Behav Pediatr. 2010;31(3):192-201.

[8] Kumari M, Ticku MK. Regulation of NMDA receptors by ethanol. Prog Drug Res. 2000;54:152-189.

[9] Deng J, Elberger AJ. Corpus callosum and visual cortex of mice with deletion of the NMDA-NR1 receptor II. Attenua-

tion of prenatal alcohol exposure effects. Brain Res Dev Brain Res. 2003;144(2):135-150.

[10] He Q, Titley H, Grasselli G, et al. Ethanol affects NMDA receptor signaling at climbing fiber-Purkinje cell synapses in mice and impairs cerebellar LTD. J Neurophysiol. 2013; 109(5):1333-1342.

[11] Naseer MI, Lee HY, Ullah N, et al. siRNA-mediated GABA(B) receptor at early fetal rat brain upon acute and chronic ethanol exposure: Down regulation of PKA and p-CREB expression. Synapse. 2011;65(2):109-118.

[12] Villas Boas GR, Zamboni CG, Peretti MC, et al. GABA (B) receptor agonist only reduces ethanol drinking in light-drinking mice. Pharmacol Biochem Behav. 2012; 102(2):233-240.

[13] Gonzalez C, Moss SJ, Olsen RW. Ethanol promotes clathrin adaptor-mediated endocytosis via the intracellular domain of δ-containing GABAA receptors. J Neurosci. 2012;32(49):17874-17881.

[14] Smith SM. Alcohol and cell death. Comprehensive Toxicology, 2nd ed. Madison: University of Wisconsin-Madison, USA. 2010.

[15] Schatter B, Jin S, Löffelholz K, et al. Cross-talk between phosphatidic acid and ceramide during ethanol-induced apoptosis in astrocytes. BMC Pharmacol. 2005;5:3.

[16] Saito M, Saito M, Cooper TB, et al. Ethanol-induced changes in the content of triglycerides, ceramides, and glucosylceramides in cultured neurons. Alcohol Clin Exp Res. 2005;29(8):1374-1383.

[17] Buccoliero R, Futerman AH. The roles of ceramide and complex sphingolipids in neuronal cell function. Pharmacol Res. 2003;47(5):409-419.

[18] Reynolds CP, Maurer BJ, Kolesnick RN. Ceramide synthesis and metabolism as a target for cancer therapy. Cancer Lett. 2004;206(2):169-180.

[19] Holthuis J, Vacaru A, Tafesse FG, et al. Sphingomyelin synthases: double trouble in the family. Chem Phys Lipids. 2007;149(Suppl 1):S8.

[20] Subathra M, Qureshi A, Luberto C. Sphingomyelin synthases regulate protein trafficking and secretion. PLoS One. 2011;6(9):e23644.

[21] Asano S, Kitatani K, Taniguchi M, et al. Regulation of cell migration by sphingomyelin synthases: sphingomyelin in lipid rafts decreases responsiveness to signaling by the CXCL12/CXCR4 pathway. Mol Cell Biol. 2012;32(16): 3242-3252.

[22] Tafesse FG, Huitema K, Hermansson M, et al. Both sphingomyelin synthases SMS1 and SMS2 are required for sphingomyelin homeostasis and growth in human HeLa cells. J Biol Chem. 2007;282(24):17537-17547.

[23] Scharfman HE. Neurogenesis in the dentate gyrus and its potential role in epileptogenesis. Encyclopedia of Basic Epilepsy Research. Oxford: Academic Press, UK. 2009.

[24] Francis F, Koulakoff A, Boucher D, et al. Doublecortin is a developmentally regulated, microtubule associated protein expressed in migrating and differentiating neurons. Neuron. 1999;23(2):247-256.

[25] Gangoiti P, Granado MH, Arana L, et al. Activation of protein kinase C α is essential for stimulation of cell proliferation by ceramide-1-phosphate. FEBS Lett. 2010; 584(3):517-524.

[26] Milotová M, Riljak V, Jandová K, et al. Changes of hippocampal neurons after perinatal exposure to ethanol. Physiol Res. 2008;57(2):275-282.

[27] Sabeti J. Ethanol exposure in early adolescence inhibits intrinsic neuronal plasticity via sigma-1 receptor activation in hippocampal CA1 neurons. Alcohol Clin Exp Res. 2011; 35(5):885-904.

[28] Tanchuck MA, Yoneyama N, Ford MM, et al. Assessment of GABA-B, metabotropic glutamate, and opioid receptor involvement in an animal model of binge drinking. Alcohol. 2011;45(1):33-44.

[29] Taslim N, Saeed Dar M. The role of nicotinic acetylcholine receptor (nAChR) α7 subtype in the functional interaction between nicotine and ethanol in mouse cerebellum. Alcohol Clin Exp Res. 2011;35(3):540-549.

[30] Delis F, Thanos PK, Rombola C, et al. Chronic mild stress increases alcohol intake in mice with low dopamine D2 receptor levels. Behav Neurosci. 2013;127(1):95-105.

[31] Lee YJ, Shukla SD. Pro- and anti-apoptotic roles of c-Jun N-terminal kinase (JNK) in ethanol and acetaldehyde exposed rat hepatocytes. Eur J Pharmacol. 2005;508(1-3): 31-45.

[32] Young C, Roth KA, Klocke BJ, et al. Role of caspase-3 in ethanol-induced developmental neurodegeneration. Neurobiol Dis. 2005; 20(2):608-614.

[33] Saito M, Chakraborty G, Hegde M, et al. Involvement of ceramide in ethanol-induced apoptotic neurodegeneration in the neonatal mouse brain. J Neurochem. 2010; 115(1):168-177.

[34] Liu J, Huan C, Chakraborty M, et al. Macrophage sphingomyelin synthase 2 deficiency decreases atherosclerosis in mice. Circ Res. 2009;105(3):295-303.

[35] Tafesse FG, Ternes P, Holthuis JC. The multigenic sphingomyelin synthase family. J Biol Chem. 2006;281(40): 29421-29425.

[36] Holthuis JC, Luberto C. Tales and mysteries of the enigmatic sphingomyelin synthase family. Adv Exp Med Biol. 2010;688:72-85.

[37] Milhas D, Clarke CJ, Hannun YA. Sphingomyelin metabolism at the plasma membrane: implications for bioactive sphingolipids. FEBS Lett. 2010;584(9):1887-1894.

[38] Meng X, Wang H, He Y, et al. A MAPK cascade downstream of ERECTA receptor-like protein kinase regulates Arabidopsis inflorescence architecture by promoting localized cell proliferation. Plant Cell. 2012;24(12):4948- 4960.

[39] Zheng H, Ying H, Wiedemeyer R, et al. PLAGL2 regulates Wnt signaling to impede differentiation in neural stem cells and gliomas. Cancer Cell. 2010;17(5):497-509.

[40] Sun J, Zhou W, Sha B, et al. Ischemia induced neural stem cell proliferation and differentiation in neonatal rat involved vascular endothelial growth factor and transforming growth factor-beta pathways. Brain Dev. 2010;

32(3):191-200.
[41] Wang Y, Martin JF, Bai CB. Direct and indirect requirements of Shh/Gli signaling in early pituitary development. Dev Biol. 2010;348(2):199-209.
[42] Riboni L, Bassi R, Anelli V, et al. Metabolic formation of ceramide-1-phosphate in cerebellar granule cells: evidence for the phosphorylation of ceramide by different metabolic pathways. Neurochem Res. 2002;27(7-8):711-716.
[43] van Echten-Deckert G, Herget T. Sphingolipid metabolism in neural cells. Biochim Biophys Acta. 2006;1758(12):1978-1994.
[44] Krause WJ, Saunders NR. Brain growth and neocortical development in the opossum. Ann Anat. 1994;176(5):395-407.
[45] Jiang Q, Hu Y, Wu P, et al. Prenatal alcohol exposure and the neuroapoptosis with long-term effect in visual cortex of mice. Alcohol Alcohol. 2007;42(4):285-290.
[46] Kuhn HG, Palmer TD, Fuchs E. Adult neurogenesis: a compensatory mechanism for neuronal damage. Eur Arch Psychiatry Clin Neurosci. 2001;251(4):152-158.
[47] Chambard JC, Lefloch R, Pouysségur J, et al. ERK implication in cell cycle regulation. Biochim Biophys Acta. 2007;1773(8):1299-1310.
[48] Granado MH, Gangoiti P, Ouro A, et al. Ceramide-1-phosphate (C1P) promotes cell migration Involvement of a specific C1P receptor. Cell Signal. 2009;21(3):405-412.
[49] Brunet A, Datta SR, Greenberg ME. Transcription-dependent and -independent control of neuronal survival by the PI3K/Akt signaling pathway. Curr Opin Neurobiol. 2001;11(3):297-305.
[50] Gomez-Muñoz A, Duffy PA, Martin A, et al. Short-chain ceramide-1-phosphates are novel stimulators of DNA synthesis and cell division: antagonism by cell-permeable ceramides. Mol Pharmacol. 1995;47(5):833-839.
[51] Gangoiti P, Granado MH, Wang SW, et al. Ceramide-1-phosphate stimulates macrophage proliferation through activation of the PI3-kinase/PKB, JNK and ERK1/2 pathways. Cell Signal. 2008;20(4):726-736.
[52] Huang HW, Goldberg EM, Zidovetzki R. Ceramides modulate protein kinase C activity and perturb the structure of phosphatidylcholine/phosphatidylserine bilayers. Biophys J. 1999;77(3):1489-1497.
[53] Hinkovska-Galcheva V, VanWay SM, Shanley TP, et al. The role of sphingosine-1-phosphate and ceramide-1-phosphate in calcium homeostasis. Curr Opin Investig Drugs. 2008;9(11):1192-1205.
[54] Shimizu M, Tada E, Makiyama T, et al. Effects of ceramide, ceramidase inhibition and expression of ceramide kinase on cytosolic phospholipase A2alpha; additional role of ceramide-1-phosphate in phosphorylation and Ca^{2+} signaling. Cell Signal. 2009;21(3):440-447.
[55] Bornancin F, Parker PJ. Phosphorylation of protein kinase C-alpha on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state. J Biol Chem. 1997;272(6):3544-3549.
[56] Li Z, Hailemariam TK, Zhou H, et al. Inhibition of sphingomyelin synthase (SMS) affects intracellular sphingomyelin accumulation and plasma membrane lipid organization. Biochim Biophys Acta. 2007;1771(9):1186-1194.
[57] National Research Council (US). Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington (DC): National Academies Press, USA. 2011.
[58] Hojjati MR, Jiang XC. Rapid, specific, and sensitive measurements of plasma sphingomyelin and phosphatidylcholine. J Lipid Res. 2006;47(3):673-676.
[59] Blakesley RW, Boezi JA. A new staining technique for proteins in polyacrylamide gels using coomassie brilliant blue G250. Anal Biochem. 1977;82(2):580-582.

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