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N-Docosahexaenoylethanolamine ameliorates ethanol-induced impairment of neural stem cell neurogenic differentiation

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Abstract

Previous studies demonstrated that prenatal exposure to ethanol interferes with embryonic and fetal development, and causes abnormal neurodevelopment. Docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid highly enriched in the brain, was shown to be essential for proper brain development and function. Recently, we found that *N*-docosahexenoyethanolamine (synaptamide), an endogenous metabolite of DHA, is a potent PKA-dependent neurogenic factor for neural stem cell (NSC) differentiation. In this study, we demonstrate that ethanol at pharmacologically relevant concentrations down regulates cAMP signaling in NSC and impairs neurogenic differentiation. In contrast, synaptamide reverses ethanol-impaired NSC neurogenic differentiation through counter-acting on the cAMP production system. NSC exposure to ethanol (25-50 mM) for 4 days dose-dependently decreased the number of Tuj-1 positive neurons and PKA/CREB phosphorylation with a concomitant reduction of cellular cAMP. Ethanol-induced cAMP reduction was accompanied by the inhibition of G-protein activation and expression of adenylyl cyclase (AC) 7 and AC8, as well as PDE4 upregulation. In contrast to ethanol, synaptamide increased cAMP production, GTPγS binding, and expression of AC7 and AC8 isoforms in a cAMP-dependent manner, offsetting the ethanol-induced impairment in neurogenic differentiation. These results indicate that synaptamide can reduce ethanol-induced impairment of neuronal differentiation by counter-affecting shared targets in G-protein coupled receptor (GPCR)/cAMP signaling. The synaptamide-mediated mechanism observed in this study may offer a possible avenue for ameliorating the adverse impact of fetal alcohol exposure on neurodevelopment.

Graphical abstract

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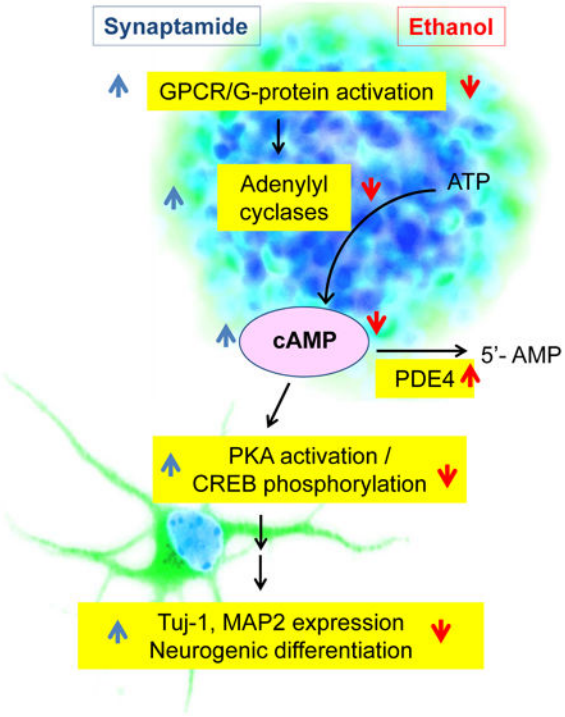

Keywords

Synaptamide; neurogenesis; cAMP; adenylyl cyclase; docosahexaenoic acid (DHA, 22:6n-3); G-protein

1. Introduction

Prenatal exposure to ethanol can adversely affect offspring neurodevelopment causing lasting deficits in brain function (Sampson et al., 1997). Neurogenesis, differentiation and neuronal survival are among key neurobiological processes inhibited by ethanol during development (Geil et al., 2014; Ikonomidou et al., 2000; Miller, 1996; Pierce et al., 1993). In rodent models, ethanol intake has been shown to inhibit the proliferation of neural precursor or stem cells in developing and adolescent brains (Miller, 1996; Morris et al., 2010) as well as adult hippocampal neurogenesis (Geil et al., 2014; Nixon and Crews, 2002). Ethanol also promoted neuronal cell death in several regions of developing brains (Ikonomidou et al., 2000; Morris et al., 2010; Olney JW1, 2002), which, together with inhibited neurogenesis, contributed to the reduction of neuron numbers (Jacobs and Miller, 2001). Recently, ethanol was shown to influence the differentiation trajectory of embryonic stem cells, reducing the number of cells with neuronal lineage (Sanchez-Alvarez et al., 2013).

Cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB) signaling has been implicated in neurogenesis, differentiation and neuronal connectivity and plasticity (Dworkin and Mantamadiotis, 2010; Lepski et al., 2011; Lonze and Ginty, 2002; Nakagawa et al., 2002; Nicol and Gaspar, 2014). Blocking cAMP/CREB signaling during embryogenesis has been shown to cause abnormal brain development (Rudolph et al., 1998;

Struthers, 1991). Ethanol significantly perturbed the adenylyl cyclase (AC) system and cAMP/CREB signaling, and thus affected a multitude of biological processes involved in neurodevelopment (Geil et al., 2014; Moonat et al., 2010; Tabakoff and Hoffman, 1998). While acute ethanol exposure enhanced the agonist-stimulated AC catalytic activity, chronic exposure to ethanol produced adaptive changes in AC function both in animals and in cell cultures (Gobejishvili et al., 2006; Hoffman and Tabakoff, 1990; Rabin, 1990). Desensitization of the AC system by long-term exposure to ethanol is heterologous, as the diminished AC activity was observed in response to a variety of activators (Gordon et al., 1986; Richelson et al., 1986; Tabakoff et al., 1995). Despite parallel observations of inhibited neurogenesis and AC system downregulation caused by chronic ethanol exposure, a direct link between ethanol-induced cAMP regulation and neurogenic differentiation has not been clearly established.

N-docosahexaenoylethanolamine (synaptamide), an endogenous metabolite of docosahexaenoic acid (DHA, 22:6n-3) with an endocannabinoid-like structure (Kim and Spector, 2013), is a potent mediator for neurite growth, synaptogenesis and neurogenic differentiation of neural stem cells (NSCs) (Kim et al., 2011; Rashid et al., 2013). Metabolism of DHA to synaptamide has been shown to occur in NSCs, neuron cultures and brain homogenates, and the synaptamide level in the brain was modulated by diet (Kim et al., 2011). The synaptamide effect on neurogenic differentiation was mediated through PKA/CREB signaling which is also an inhibitory target for chronic ethanol exposure. In this study, we first established the effects of chronic ethanol on neurogenic differentiation in relation to cAMP regulation. Since ethanol and synaptamide counter-affect cAMP/PKA/CREB signaling, we subsequently investigated possible interaction between synaptamide and ethanol on neurogenic differentiation of NSCs. The current investigation revealed that ethanol inhibits cAMP/PKC/CREB signaling and neuronal differentiation of NSCs, while DHA-derived synaptamide reverses these ethanol effects through potent activation of cAMP-dependent signal transduction.

2. Materials and methods

2.1 Chemicals and antibodies

Dulbecco's modified Eagle's medium/Ham'a F12 (DMEM/F12 1 : 1) and insulin were acquired from Life Technologies Corporation (Carlsbad, CA, USA) and basic fibroblast growth factor (bFGF) from R&D Systems (Minneapolis, MN, USA). Glucose, HEPES, human apo-transferrin, progesterone, sodium selenite, putrescine, paraformaldehyde, heparin, Poly-L-ornithine, forskolin, caffeine, rolipram and SQ 22536 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to PKA, CREB, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling (Danvers, MA, USA), and Tuj-1 antibody was acquired from EMD Millipore (Billerica, MA, USA). Adenylyl cyclase 7 (AC7) and Anti-G protein alpha S antibodies were purchased from Abcam (Cambridge, MA, USA), AC1, AC2, AC4, and AC8 antibodies were from Santa Cruz Biotechnology (Dalla, TX, USA) and PDE4B antibody was purchased from Novus Biologicals (Littleton, CO, USA). Free fatty acids were obtained from Nuchek Prep Inc.

(Elysian, MN, USA). Synaptamide was prepared from DHA-chloride (Nu-Check Prep) and ethanolamine (Sigma) as described earlier (Kim et al. 2011).

2.2 Animals

Timed pregnant female Wistar rats (gestation day 12) were acquired from Charles River Laboratories (Portage, MI, USA) and adjusted for a day before fetal brains were collected for NSC preparation. All experiments in this study were carried out in accordance with the guiding principles for the care and use of animals approved by the National Institute on Alcohol Abuse and Alcoholism (LMS-HK-13, LMS-HK-41). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques if available.

2.3 Fetal neural stem cell culture

NSCs were cultured by the neurosphere method as described by (Rashid et al., 2013). Briefly, forebrain cortices of fetal brains were isolated on embryonic day 14.5. The cortices were mechanically disrupted into single cells by repeated pipetting in a serum-free conditioned medium (N2 medium) containing DMEM/F12 (1 : 1), 0.6% (wt/vol) glucose, 0.1125% (wt/vol) sodium bicarbonate, 2 mM L-glutamine, 5 mM HEPES, 100 μg/mL human apo-transferrin, 20 nM progesterone, 30 nM sodium selenite, 60 μM putrescine, and 25 μg/mL insulin. The dissociated cells were cultured in 6 cm dishes at a density of 1×10^5 cells/mL in N2 medium with 20 ng/mL bFGF and 2 μg/mL heparin in a humidified 5% CO2/95% air incubator at 37°C. Within 3–4 days, the cells grew as free floating neurospheres that were then collected by centrifugation, mechanically dissociated by pipetting, and passaged. After the second passage, nestin- and SOX2-positive NSCs were enriched in neurospheres with minimal presence of differentiated cells (MAP2-, Tuj-1, and GFAP-positive cells). The NSCs which were dissociated from the neurospheres and cultured for 3 h in the absence of FGF were mostly nestin- and SOX2-positive, indicating the stemness of the NSC preparation.

2.4 Differentiation of NSCs and treatment with ethanol and synaptamide

After the second passage neurospheres were mechanically dissociated and 5×10^5 cells/mL were plated onto 15 μg/mL poly-L-ornithine-coated 6- or 24-well plates in N2 medium without bFGF and heparin to initiate the differentiation. To evaluate the synaptamide effects NSC cultures were treated with N2 medium containing synaptamide bound to 0.05% (wt/vol) bovine serum albumin (BSA) and 40 μM vitamin E for 3-4 days as described earlier (Rashid et al., 2013). As the vehicle control, N2 medium containing 0.05% (wt/vol) BSA and 40 μM vitamin E was used. To generate the NSCs chronically exposed to ethanol, NSCs were cultured in media containing 25-50 mM ethanol by changing the ethanol-containing media daily for 4 days. For acute ethanol treatment, NSCs were treated with 0-50 mM ethanol for 15 min, 1 h or 6 h, before cellular cAMP production was evaluated by ELISA.

2.5 Immunofluorescence staining

For immunofluorescence staining, 2.5×10^5 NSCs were cultured in 0.5 mL media unless otherwise specified. Cultured cells were fixed with 4% (wt/vol) paraformaldehyde for 30

min at 25°C, washed with 0.1 M Tris-buffered saline (pH 7.5, TBS), blocked with 10% (vol/vol) normal goat serum in TBS containing 0.3% (vol/vol) Triton X-100 at 25°C for 60 min, and incubated with mouse anti-Tuj-1 (1: 1000) primary antibody at 4°C overnight. The cells were washed with TBS and incubated with Alexa Fluor 488-conjugated secondary antibodies (1:1000, Life Technologies Corporation) at 25°C for 60 min. To visualize nuclei, the cells were counter-stained with 2 μg/mL 4′,6-diamidino-2-phenylindole (DAPI). Finally, the cells were mounted with 80% (vol/vol) glycerol, visualized under a fluorescent microscope (IX81; Olympus Corp., Tokyo, Japan) and the image data were processed using MetaMorph (Molecular Devices, Sunnyvale, CA, USA) for quantitative information. The number of Tuj-1 positive cells was counted from three separate wells with twelve to fifteen random fields per well for each individual experiment. At least three independent experiments were performed. The percentage of Tuj-1-positive neuronal cell population was calculated against the DAPI-positive total cell numbers which include undifferentiated stem cells and differentiated neuronal cells.

2.6 cAMP assay

For cAMP assay, 2.5×10^5 NSCs cells were cultured in 0.5 mL media unless specified otherwise. Cultured NSCs (DIV4) were treated with synaptamide for 15 min with or without the 30 min pretreatment with 100 μM AC inhibitor SQ 22536, 10 μM rolipram or 10 μM caffeine. cAMP levels were determined using cyclicAMP XP® assay kit (Cell signaling, Danvers, MA) according to the manufacturer's protocol. Briefly, cells were lysed using a lysis buffer including protease inhibitor cocktail (Cell Signaling) and the cell lysate was added to the cyclicAMP XP® assay kit to displace HRP-linked cAMP bound to an anti-cAMP XP® Rabbit mAb immobilized onto a 96-well plate. After removing displaced HRP-linked cAMP, HRP substrate TMB was added and cAMP concentration was measured colorimetrically at 450 nm.

2.7 RNA isolation and quantitative RT-PCR

Total RNA was extracted from rat cortices NSCs culture using Trizol according to manufacturer's protocol (Invitrogen, UK). RNA was treated with DNase I to remove any contaminating genomic DNA (RQ1 RNase-Free DNase; Promega). RNA was then used for cDNA synthesis applying reverse transcription reagents (Applied Biosystems Inc., Foster City, CA, USA). Expression of mRNA for Gnas (NM_001024823), Adcy1 (NM_001107239), Adcy2 (NM_031007), Adcy3 (NM_130779), Adcy4 (NM_019285), Adcy6 (NM_012821), Adcy7 (NM_053396), Adcy8 (NM_017142), PDE4A (NM_013101), PDE4B (NM_017031), PDE4C (XM_001070301), PDE4D (NM_001113328), and GAPDH (NM_017008) were measured via a TaqMan-based real-time RT-PCR assay. Samples were analyzed in triplicate on an ABI Prism 7900HT sequence detection system and PerfeCTa SYBR Green FastMix, Low ROX reagents (Quanta Biosciences). The amplification conditions were 50°C for 2 min then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The SDS 2.0 software (Applied Biosystems Inc., Foster City, CA, USA) was used to analyze and convert the expression data into cycle threshold values (Ct-values). The relative expression of mRNA was calculated after normalization to GAPDH mRNA.(Griffin et al., 2000)

2.8 Neural stem cell membrane preparation

Neural stem cell plasma membrane was prepared as previously described (Griffin et al., 2000) with minor modification. Briefly, NSCs (12.5×10^6) cultured in 25 mL media were harvested in phosphate-buffered saline (PBS) containing 1 mM EDTA, and centrifuged at 1,000 × g for 10 min at 4 °C. Cells were then suspended and homogenized in ice cold homogenization buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, pH 7.4, protease inhibitor cocktail), centrifuged at 1,000 × g for 10 min at 4 °C. The supernatant was collected and the cell pellet was resuspended in the homogenization buffer and the homogenization process was repeated twice. After the supernatants were combined and centrifuged again at 48,000 × g for 30 min, the pellet was suspended in ice-cold TME-Na buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4, protease inhibitor cocktail). The protein content of the membrane fraction was determined by the biccinchoninic acid method (Smith et al., 1985), using bovine serum albumin (BSA) as the standard.

2.9 [γ^{35} S]-GTP assay in NSC membranes

[γ^{35}S]-GTP binding assay was performed as previously described (Gonzalez-Maeso et al., 2000) with minor modification. Briefly, the assay buffer (50 mM Tris-HCl, 3mM $MgCl_2$, 100 mM NaCl, 0.2 mM EGTA) was mixed with 3 μM GDP, 0.08 nM [^{35}]GTPγS and synaptamide (10 nM) in silicone-treated test tubes. The reaction was initiated by adding NSC membranes (30 μg protein) to the assay buffer prepared above in a final volume of 500 μL, and the reaction mixture was incubated for 1 h at 30 °C. The reaction was terminated by diluting the reaction mixture with 100 μL of ice-cold stopping buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM $MgCl_2$, 1 mM EGTA, 1% cholate), and filtered over nitrocellulose membranes on a vacuum manifold. The nitrocellulose membranes were then washed 3 times with 2 mL of ice-cold wash buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM $MgCl_2$, 1 mM EGTA), dried and added 5 mL of Econo-Safe scintillation cocktail (Beckman Coulter, USA). The membrane-bound radioactivity was measured by liquid scintillation (Beckman Coulter LS 6500, USA) at efficiency of 95% for ^{35}S. The basal activity was estimated in the absence of synaptamide.

2.10 Western blot analysis

For Western blot analysis, 1.25×10^6 NSCs were cultured in 2.5 mL media unless specified otherwise. Proteins in cell lysates (20 μg protein) were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride (PVDF) membrane for 90 min at 100 V at 4 °C. The membranes were blocked with 5% BSA for 60 min in TBST (20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.1% (vol/vol) Tween 20), and incubated overnight at 4°C with primary antibodies diluted (1:1000) in TBST. After being washed in TBST buffer, the membranes were incubated for 60 min in anti-rabbit or anti-mouse IgG–horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) secondary antibodies diluted (1:2000) in TBST, and the labeled proteins were detected with chemiluminescence reagents (Thermo Fisher Scientific, Rockford, IL, USA). Experiments were repeated three times. Western blots intensities of bands were

quantitated using Image J software (NIH, Bethesda, MD) as previously described (Kwon et al., 2011).

2.11 Cytotoxicity assay

To evaluate cytotoxicity, lactate dehydrogenase (LDH) released from NSCs was assayed using CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. NSCs (2.5×10^5 NSCs in 0.5 mL media) were treated with ethanol (25-100 mM) containing media for 4 days, and then incubated with synaptamide (10 nM) for additional 3 days in corresponding media. The 50 μL supernatant solution was collected from the culture, transferred to 96-well plates, and 50 μL of substrate solution was added. The enzymatic reaction was allowed to proceed for 30 min at 25°C, protected from light. After stopping the reaction by adding 50 μL/well of the stop solution, the absorbance was measured at 490 nm using a plate reader (Molecular Devices). The released LDH activity was normalized to the total LDH activity determined from the cell lysate and supernatant. The final data were expressed as the % of untreated control.

2.12 Ethanol assay

C57BL6/J wild type mice were given ethanol (1-2.5 gm/kg body weight) via oral gavage and mandibular (facial vein/artery) blood samples were taken at 0, 30, 60 min after ethanol gavage. The blood ethanol level was determined using an Ethanol assay kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

2.13 Statistical analysis

The results are expressed as means ± SD for triplicate samples and represent at least three independent experiments. Statistical analyses were conducted using Student's t-test and in some cases two-way ANOVA with Bonferroni's multiple comparisons test, using PRISM 6 software (GraphPad, San Diego, CA). Unless indicated otherwise, a p-value of less than 0.05 was considered significant.

3. Results

3.1 Ethanol decreases cAMP production and neurogenic differentiation of NSCs

Previous studies have shown that chronic ethanol exposure during neurodevelopment impairs cAMP production and phosphorylation of PKA/CREB *in vitro* and *in vivo* (Gobejishvili et al., 2006; Misra and Pandey, 2003; Yang et al., 1998). Prior to evaluating whether chronic ethanol exposure has an impact on neurogenic differentiation, we first examined the role of cAMP increase in neurogenic differentiation of NSCs (Fig. 1). We previously found that forskolin, which activates adenylate cyclase and thus raises the cAMP level, significantly increased neurogenic differentiation (Rashid et al., 2013). When cAMP production was blocked by SQ22536, an AC inhibitor, forskolin-induced neurogenic differentiation was no longer observed (Fig. 1A, B), indicating that the observed increase in neurogenesis was due to the upregulation of the cAMP production. This result indicates that cAMP production can serve as an indicator for the neurogenic potential of NSCs. Despite a decreasing trend, acute or short-term ethanol treatment at pharmacologically relevant concentrations (25 to 50 mM) for up to 6 h did not significantly affect the basal cAMP

production in NSCs (Fig. S1). For long term ethanol exposure, NSCs were cultured in the media containing ethanol (0-100 mM) by replenishing the medium daily for 4 days. To evaluate the effects of ethanol on the capacity to produce cAMP, the NSCs chronically exposed to ethanol were stimulated by 10 μM forskolin for 15 min and the cAMP level was determined. Four days of treatment with ethanol at pharmacologically relevant concentrations (25 to 50 mM) did not appear to exert toxic effects since no increase in the lactate dehydrogenase (LDH) activity was observed (Fig. S2). At 100 mM ethanol, LDH activity was slightly (22%) but significantly ($p<0.05$) elevated, and therefore, ethanol concentrations up to 50 mM were examined in this study. Forskolin significantly increased cAMP production (203%, $p<0.001$), and ethanol treatment dose-dependently decreased both basal and forskolin-stimulated cAMP production in differentiating NSCs (Fig. 1C). Neurogenic differentiation was subsequently evaluated by immunocytochemistry and Western blot analysis after incubating NSCs in the corresponding media for 3 additional days (Fig. 1D-F). Chronic ethanol treatment dose-dependently decreased both basal and forskolin-induced neurogenic differentiation (Fig. 1D, E). It was also apparent from the Western blot analysis that chronic ethanol reduces the expression of Tuj-1 along with phosphorylation of PKA and CREB, downstream signals of cAMP (Fig. 1F, G). These results indicate that chronic ethanol downregulates cAMP/PKA/CREB signaling and thus impairs cAMP-dependent neurogenic differentiation of NSCs.

3.2 Effect of synaptamide on ethanol-inhibited cAMP/PKA/CREB signaling and neuronal differentiation of NSCs

We have recently shown that synaptamide, an endogenous metabolite of DHA, has potent neurogenic activity via PKA/CREB phosphorylation (Rashid et al., 2013). Therefore, we examined whether this endogenous mediator can increase cAMP production and counter-act the chronic ethanol-induced impairment of cAMP production and neurogenic differentiation of NSCs. NSCs cultured in ethanol-containing media for 4 days were treated with 1-25 nM synaptamide for 15 min to evaluate the cAMP production. We found that synaptamide dose-dependently increases cellular cAMP production, reaching a plateau level at 10 nM (266% of basal, $p<0.001$) (Fig. 2A). Although the synaptamide effect was significantly dampened by ethanol, synaptamide at a concentration as low as 1-5 nM completely reversed the cAMP decrease caused by chronic exposure to 25 mM ethanol (Fig. 2A). In fact, synaptamide at 10 nM raised the cAMP level enough to completely reverse the cAMP inhibition seen after ethanol exposure at 50 mM (Fig. 2B). At 10 nM concentration, synaptamide stimulated neurogenic differentiation which was evident by increased Tuj-1-positive neurons (Fig. 2C, D) as well as the increased protein levels of Tuj-1 and phosphorylated PKA and CREB (Fig. 2E, F). Neurogenic differentiation impaired by ethanol even at 50 mM was rescued by 10 nM synaptamide. Attenuated Tuj-1 expression and phosphorylation of PKA and CREB due to the exposure to ethanol were also reversed by 10 nM synaptamide (Fig. 2C-F).

Since cAMP is regulated by G-protein coupled receptor (GPCR) signaling, we also examined the effect of chronic ethanol and synaptamide on G-protein activation. Exchange of GDP with a hydrolysis-resistant ^{35}S-labelled GTP analogue [γ-^{35}S] GTP was measured in membranes prepared from control or ethanol-exposed NSCs (Fig. 2G). We found that the basal [γ-^{35}S] GTP binding is slightly but significantly decreased (19% decrease, $p<0.05$) in

membranes from NSCs chronically exposed to 50 mM ethanol. When non-ethanol treated control membranes were stimulated with 10 nM synaptamide, GDP/GTP exchange activity increased significantly (184%, $p<0.01$), indicating that synaptamide action is mediated by GPCR signaling. The synaptamide-induced increase in GDP/GTP exchange was reduced in membranes from ethanol-exposed NSCs (184% vs. 137%, $p<0.05$). Nevertheless, the exchange activity after synaptamide treatment was still significantly higher than that of the non-ethanol-treated control. These results indicate that synaptamide can ameliorate chronic ethanol-induced impairment of neurogenic differentiation through activating a GPCR and augmenting cAMP/PKA/CREB signaling.

3.3 Potential targets for ethanol-impaired neurogenic differentiation of NSCs

Adenylyl cyclases (ACs) and phosphodiesterases (PDEs) which synthesize and hydrolyze cAMP, respectively, play a crucial role in modulating intracellular cAMP levels (Houslay and Adams, 2003; Huang et al., 2013). Therefore, we examined the possibility of ACs and PDEs as potential targets for ethanol and synaptamide action using specific inhibitors (Fig. 3A). After NSCs cultured in ethanol-containing media for 4 days were pretreated with the AC inhibitor (SQ 22536) or non-selective (caffeine) and selective PDE inhibitors (rolipram) for 30 min, cAMP production was evaluated under basal and synaptamide-stimulated conditions. The basal cAMP level was not significantly altered by SQ22536, but increased significantly when cAMP degradation by PDE was inhibited by caffeine or rolipram, a selective inhibitor of PDE4 which is the predominant PDE protein expressed in brain. This observation indicates that PDE rather than AC activity contributes more to the basal cAMP maintenance in NSCs. Synaptamide-induced cAMP production was significantly dampened by SQ22536 but potentiated by caffeine or rolipram. A dose-dependent decrease of cAMP by chronic ethanol treatment was observed in all cases, regardless of potentiated (with synaptamide or PDE4 inhibitors) or suppressed (with AC inhibitor) conditions. The ability of ethanol to lower cAMP levels remained in the absence of either AC or PDE activity, suggesting that ethanol acts on both ACs and PDE4.

The inhibitor effects on neurogenic differentiation were similar to the cAMP profile. AC inhibition by SQ22536 did not affect basal differentiation but significantly decreased Tuj-1-positive neurons in synaptamide-treated NSCs. In contrast, rolipram significantly increased Tuj-1-positive neurons under both basal and synaptamide-stimulated conditions. Chronic ethanol treatment dose-dependently decreased Tuj-1-positive neurons in all conditions examined (Fig. 3B, Fig. S3), confirming that ethanol suppresses neurogenic differentiation through affecting both ACs and PDE4. The two-way ANOVA data indicates a significant interaction ($p=0.0001$) between the synaptamide and ethanol effects on both cAMP production (Supplemental Table 1) and neurogenic differentiation (Supplemental Table 2), indicating that synaptamide and ethanol act on common targets. For better comparison, a two-way ANOVA was also performed for cAMP production (Fig. 3A) and neurogenic differentiation (Fig. 3B) and the data are presented as Supplemental Tables 3 and 4, respectively. The inhibition profile together with the [γ-^{35}S] GTP binding data (shown in Fig. 2G) suggest that synaptamide ameliorates the ethanol-induced impairment of cAMP production and neurogenic differentiation at least partly through offsetting the adverse impact of ethanol on G-protein activation, AC and PDE4.

3.4 Effect of ethanol and synaptamide on Gα, PDE4 and ACs

The data indicating that chronic ethanol exposure and synaptamide have multiple common targets in G-protein signaling suggest possible alterations in the expression of central proteins that regulate intracellular cAMP. Therefore, the expression of those proteins was examined using Western blot analysis and/or real time PCR (Fig. 4). In addition to ACs and PDEs, effects of ethanol on Gαs was also examined since ethanol inhibited G-protein activation with concomitant cAMP reduction under both basal and synaptamide-stimulated conditions, suggesting likely involvement of Gαs protein downregulation. However, mRNA or protein expression of Gαs was unaltered by either four days of ethanol exposure or synaptamide treatment for 24 h (Fig. 4A,B), suggesting that G-protein activation inhibited by chronic ethanol treatment may involve target events other than Gαs expression. The chronic ethanol treatment elevated cAMP-specific PDE4B protein (Fig. 4A). However, the mRNA level of any of the PDE4 isoforms was unaltered (Fig. 4C), suggesting a role of ethanol in stabilizing PDE4B protein. The synaptamide treatment showed no effects on either PDE4B protein levels (Fig. 4A) or the mRNA expression of PDE4 isoforms at 6 h (data not shown) or 24 h after treatment (Fig. 4C).

Among the AC isoforms examined for mRNA expression, only AC7 and AC8 were sensitive to the treatment with chronic ethanol or synaptamide (Fig. 5A). The mRNA expression of AC7 and AC8 was decreased by ethanol dose-dependently after four days of exposure. In contrast, 10 nM synaptamide treatments for 24 h significantly increased their expression. Although the extent of the synaptamide-mediated mRNA increase for AC7 and AC8 was less in ethanol-treated NSCs, synaptamide prevented ethanol-induced reduction of these AC isoforms below their basal level. The protein level of AC7 and AC8 followed a similar pattern (Fig. 5B) in that chronic ethanol decreased AC7 and AC8 while synaptamide alleviated their reduction caused by ethanol.

Interestingly, the AC7 and AC8 expression modulated by synaptamide was dependent on cAMP production, as the AC inhibitor SQ prevented the synaptamide-induced upregulation of these isoforms (Fig. 6A-C). The upregulated AC proteins (Fig. 6D) appeared functional as forskolin-stimulated cAMP production in the synaptamide-primed cells was significantly higher compared to non-synaptamide-primed control (Fig. 6E). Since NSCs were washed prior to the stimulation, the basal cAMP production during 15 min vehicle treatment did not reflect the effect of significantly elevated AC7 and AC8 in synaptamide-primed NSCs. These results indicate that synaptamide counter-affects the negative impact of ethanol on cAMP production at least in part through G-protein activation and induction of AC7 and AC8.

4. Discussion

In this study, we demonstrate for the first time that an omega-3 fatty acid-derived metabolite offsets the adverse impact of chronic ethanol exposure on neurogenic differentiation of neural stem cells. Ethanol and synaptamide have opposite effects on cAMP signaling, particularly G-protein activation and AC expression, affecting cAMP-dependent neurogenic differentiation of NSCs. These findings suggest that synaptamide can ameliorate ethanol-

induced impairment of neurogenesis in developing brains. The counter-action of ethanol and synaptamide on shared targets in GPCR signaling is schematically depicted in Fig. 7.

Omega-3 fatty acids are found in high concentrations in neuronal membranes (Salem et al., 2001). DHA, in particular, is the most abundant omega-3 fatty acid in the brain, comprising 40% of the brain polyunsaturated fatty acids (Singh, 2005). DHA influences membrane properties, membrane-related signal transduction, synaptic transmission and receptor trafficking as well as synaptic protein expression (Akbar et al., 2005; Cao et al., 2009; Gomez-Pinilla, 2008; Sidhu et al., 2011). We have previously demonstrated that synaptamide, an endocannabinoid-like metabolite endogenously derived from DHA, potently promotes neurogenic differentiation of NSCs through PKA/CREB activation (Kim and Spector, 2013; Rashid et al., 2013). In cultured progenitor cells, activation of the cAMP pathway was shown to increase neuronal differentiation and neurite outgrowth (Palmer et al., 1997; Takahashi et al., 1999). Likewise, the current study indicates that neurogenic differentiation of NSCs is promoted by synaptamide which increased cAMP production (Fig. 2).

The molecular targets for ethanol are multiple and the effect of ethanol on the G-protein signaling is heterologous (Gordon et al., 1986; Kitanaka N, 2008; Richelson et al., 1986; Tabakoff et al., 1995). The $GABA_A$ receptor complex (Sundstrom-Poromaa I, 2002) and G-protein-coupled inwardly rectifying potassium channels (GIRKs) (Lewohl JM, 1992) are among a few of the GPCRs shown to be targeted by ethanol. Ethanol was also reported to increase GABA release for which the $G\alpha_q$- and $G\alpha_s$-coupled GPCR pathways were suggested to play a critical role (Kelm MK1, 2011). A recent study showed that monocyte-derived dendritic cells from alcohol users have higher levels of GPR55 and CNR2 encoding cannabinoid receptor 2(CB2) (Agudelo M1, 2013). Since CB2 inhibits AC through the Gi/Gα subunit, it is possible that ethanol-induced CB2 upregulation contributes to ethanol's effects on cAMP reduction. Nevertheless, specific GPCRs targeted by ethanol resulting in cAMP inhibition have yet to be demonstrated.

The intracellular levels of cAMP are regulated by the rate of synthesis and hydrolysis, which are catalyzed by AC and cAMP-specific phosphodiesterases (PDEs), respectively (Soderling and Beavo, 2000). Chronic ethanol exposure has been shown to diminish AC activity as well as cAMP accumulation (Hoffman and Tabakoff, 1990; Rabin, 1990). After chronic administration of ethanol to animals or chronic exposure of cells in culture to ethanol, the sensitivity of AC to stimulation by guanine nucleotides, hormones, and neurotransmitters decreased. For example, in neuroblastoma N1E-115 cells, chronic ethanol exposure significantly reduced the AC response to prostaglandin El (Charness et al., 1988), lowering cAMP production. Similarly, we found in this study that chronic ethanol exposure decreases cAMP production in neural stem cells. The neurogenic differentiation of NSCs was induced by forskolin and inhibited by an AC inhibitor SQ22536 (Fig.1B), indicating the cAMP-dependent nature of this biological process. Ethanol dose-dependently attenuated both cAMP production (Fig. 1C) and thus cAMP-dependent neurogenic differentiation (Fig. 1E). This ethanol effect persisted even after perturbation of the cAMP homeostasis by AC or PDE4 inhibitors (Fig. 3), suggesting that ethanol acts on multiple targets in G-protein

signaling, including AC and PDE4. Indeed, we found decreased expression of AC7 and AC8 isoforms, and elevation of PDE4B protein after chronically exposing NSCs to ethanol.

A selective effect of ethanol on cAMP synthesis by a specific AC isoform has been demonstrated using HEK293 cells transfected with different types of AC. Cells transfected with AC7 were the most sensitive to ethanol, whereas cells transfected with AC1 or AC3 were not sensitive (Yoshimura and Tabakoff, 1995, 1999). AC7 is highly concentrated in the cerebellar granule and Purkinje cell layers, and transgenic mice overexpressing AC7 specifically in the central nervous system showed behavioral and electrophysiological changes in their sensitivity to ethanol. AC8, a Ca^{2+}/calmodulin-sensitive enzyme expressed in brain regions (Cali J. J. et al., 1994; Cali et al., 1996), was also shown to be sensitive to ethanol (Chen et al., 2006). While acute ethanol treatment was shown to stimulate AC7 (Yoshimura and Tabakoff, 1995), it has been demonstrated in hypothalamic cells that chronic exposure to ethanol decreases mRNA levels of AC7 and AC8 (Chen et al., 2006). Our NSC model indicated that AC7 and AC8 are sensitive to not only ethanol but also synaptamide (Fig. 5). Similar to the previous findings, significant decreases in AC7 and AC8 were observed at both mRNA and protein levels after chronic ethanol treatment. On the contrary, we found that synaptamide increases both AC7 and AC8 expression in NSCs in a cAMP-dependent manner (Fig. 6). It has been reported that AC8 gene expression can be increased by cAMP and CREB activation, with the suggestion that its regulation may contribute to cAMP/CREB-mediated neural plasticity (Chao JR1, 2002; Schaefer et al., 2000; Wong et al., 1999). The cAMP-dependent expression of AC7 and AC8 induced by synaptamide observed in this study suggests that both isoforms may contribute to neural plasticity. In this regard, cAMP production counter-affected by ethanol and synaptamide may have significant implication in modulating various forms of cAMP-dependent neural plasticity including learning and memory, addiction and stress-induced anxiety.

PDE4 plays an important functional role in the central nervous system. Inhibition of PDE4 by rolipram enhanced brain cAMP signaling, produced antidepressant-like anxiolytic-like and memory-enhancing effects in rodents (Li et al., 2011; Li et al., 2009; Zhang, 2009) and decreased ethanol intake (Hu et al., 2011), although a definitive role of cAMP-stimulated neurogenesis in mood and memory function or drinking behavior has yet to be established. We found in this study that basal and synaptamide-stimulated cAMP production and neurogenic differentiation are significantly enhanced by rolipram, indicating an active role of PDE4 in cAMP homeostasis in NSCs. Previously, it was reported that chronic ethanol exposure increases LPS-inducible PDE4 mRNA expression in monocytes/macrophages including hepatic Kupffer cells without affecting the basal PDE4 mRNA level (Gobejishvili et al., 2008). In the same report, enhanced PDE4 activity was indicated at both basal and LPS-stimulated conditions after chronic ethanol exposure (Gobejishvili et al., 2008). Similar to their finding in the basal condition, we observed no changes in PDE4 gene expression. However, the PDE4 protein level was elevated, which likely contributed to the enhanced PDE4 activity observed previously. It is conceivable that chronic ethanol exposure may stabilize PDE4 by inhibiting degradation of PDE4. In addition to AC and PDE4 modulation, ethanol inhibited GPCR-mediated G-protein activation processes (Fig. 2G). Increases in GTP/GDP exchange activity produced by synaptamide indicate that cAMP production by synaptamide involves activation of a GPCR in differentiating NSCs. By increasing GPCR-

mediated cAMP production, synaptamide offset ethanol's multi-targeted inhibition of the cAMP signaling system and reversed impaired neurogenic differentiation of NSCs. These data demonstrate a therapeutic potential of synaptamide in neurodevelopmental damage caused by fetal alcohol exposure. Since the synaptamide level is under endogenous control, increasing its level by dietary means (Berger et al., 2001; Kim et al., 2011) may be an endogenous mechanism to enhance the offsetting effects of synaptamide on impaired neurogenesis caused by ethanol. To further validate the therapeutic potential of synaptamide, a treatment time course study is warranted, as the current study is limited to a single time point for synaptamide treatment after ethanol exposure.

In summary, chronic ethanol exposure at pharmacologically relevant concentrations impaires cAMP production and neurogenic differentiation of NSCs by affecting multiple targets in G-protein signaling such as G-protein activation, AC and PDE4. Synaptamide upregulates the cAMP signaling system and by doing so counteracts ethanol's inhibitory action on NSC differentiation. The synaptamide-dependent mechanism demonstrated in this study may provide an avenue to ameliorate some of the adverse impact of ethanol on neurodevelopmental deficits manifested in fetal alcohol spectrum disorder.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AC	Adenylyl cyclase
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
BrdU	5-Bromo-2′-deoxyuridine
NeuN	Neuronal nuclei
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element binding protein
DAPI	4′,6-diamidino-2-phenylindole
DHA	Docosahexaenoic acid
DMEM/F12	Dulbecco's modified Eagle's medium/Ham'a F12
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G protein coupled receptor
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
LDH	Lactate dehydrogenase
PDEs	Phosphodiesterases
PBS	phosphate-buffered saline
PKA	protein kinase A
NSCs	Neural stem cells
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinylidene difluoride
Tuj-1	neuron-specific class III beta-tubulin

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Highlights

- Chronic ethanol inhibits cAMP production and neurogenic differentiation of NSCs.
- Chronic ethanol downregulates G-protein/AC/cAMP signals, and upregulates PDE4.
- Synaptamide upregulates cAMP, G-protein activation and expression of AC7 and AC8.
- Synaptamide offsets ethanol-impaired neuronal differentiation.
- Synaptamide may ameliorate neurodevelopmental adversity of fetal alcohol exposure.

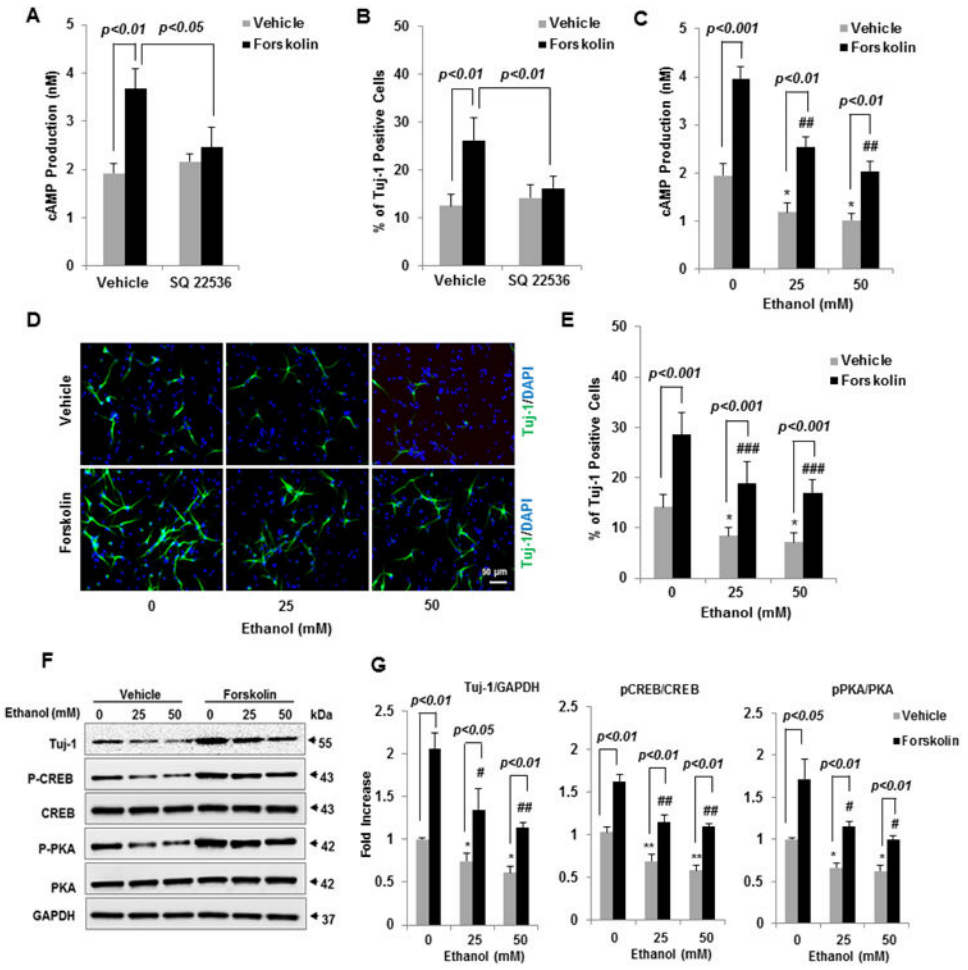

Figure 1. Inhibitory effects of ethanol on cAMP production and neuronal differentiation of NSCs

A- B: Forskolin increases cAMP production and neuronal differentiation in a cAMP-dependent manner. NSCs (DIV4) were stimulated with forskolin (10 μM) in the presence or absence of an AC inhibitor (SQ22536 100 μM) either for 15 min to determine cAMP production (A), or for 3 days to evaluate Tuj-1-positive neurons using immunocytochemistry (B). C-F: Ethanol dose-dependently inhibites cAMP production (C), neuronal differentiation (D,E) and phosphorylation of PKA and CREB (F). NSCs were chronically treated with ethanol (0-50 mM) by changing the ethanol-containing media daily for 4 days, and then stimulated with forskolin (10 μM) for 15 min for cAMP measurements (C) or for an additional 3 days in the corresponding media for evaluating neurogenic differentiation (D,E). NSCs were stained for Tuj-1 (green, early neuron marker) and nuclei (blue, DAPI), and visualized by fluorescence microscopy (D). The percentage of Tuj-1-positive cells was evaluated using MetaMorph software (B,E) and Western blot analysis was performed for CREB and PKA phosphorylation along with Tuj-1 (F), which were quantified by densitometry. Tuj-1 and phosphorylated CREB and PKA levels were normalized to GAPDH, total CREB and total PKA intensities, respectively, and the significance was determined against their basal levels (G). The percentage of Tuj-1-positive and total cells for each experiment were derived from three separate wells with twelve to fifteen random fields per well. The data are expressed as the mean ±SD of triplicates, representing three

independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared to the corresponding no-ethanol vehicle (*) or forskolin (#) control.

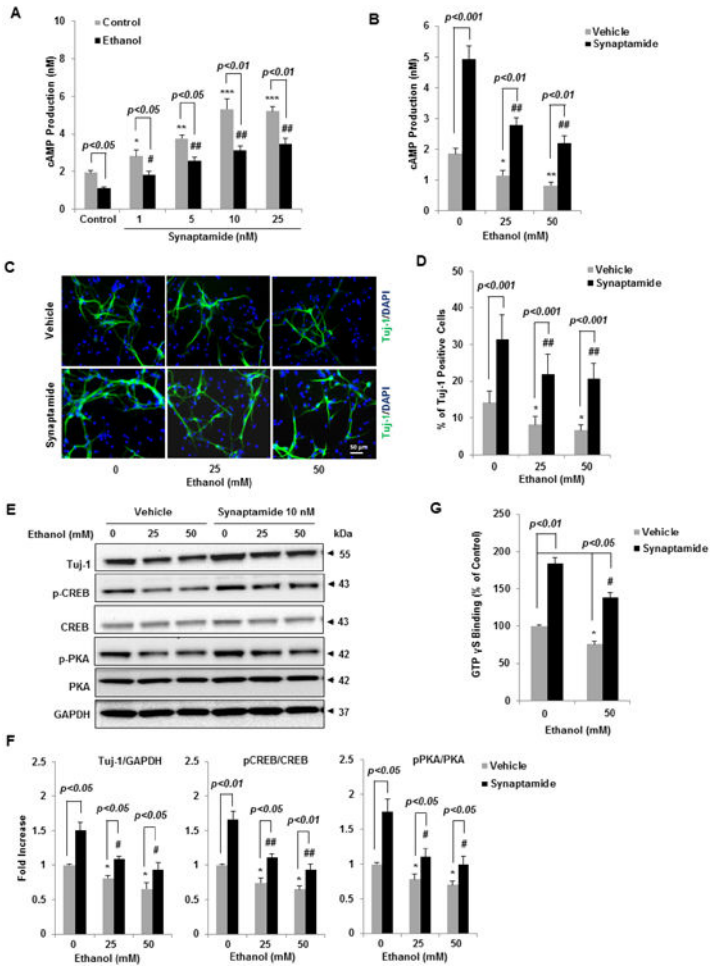

Figure 2. Effect of synaptamide on ethanol-inhibited cAMP production, G-protein activation and neuronal differentiation of NSCs

A-B: cAMP production is dose-dependently altered by synaptamide and ethanol. cAMP was measured after NSCs cultured in the media containing 25 mM ethanol for 4 days were treated with synaptamide (1-25 nM) (A), or after NSCs cultured in ethanol (0-50 mM) containing media for 4 days were treated with 10 nM synaptamide (10 nM) for 15 min (B). C-D: Ethanol-inhibited neurogenic differentiation is reversed by synaptamide. NSCs cultured in the ethanol containing media for 4 days were treated with 10 nM synaptamide and cultured for an additional 3 days in the corresponding media and the % of Tuj-1 (green, early neuron marker)-positive neurons visualized by fluorescence microscopy was evaluated against total nuclei (blue, DAPI) (C,D). The percentage of Tuj-1-positive and total cells for each experiment was derived from three separate wells with twelve to fifteen random fields per well. E. Western blot analysis of samples prepared as in C indicates dose-dependent inhibition of Tuj-1 expression and PKA/CREB phosphorylation by ethanol. F. The protein level shown in E was quantified by densitometry. Tuj-1 and phosphorylated CREB and PKA levels were normalized to GAPDH, total CREB and total PKA intensities, respectively, and the significance was determined against their basal levels G. G-protein activation is significantly stimulated by synaptamide but is inhibited by ethanol. Isolated plasma membranes from the NSCs cultured in the media containing 0 or 50 mM ethanol were

stimulated with 10 nM synaptamide for 15 min as described in the Methods section and the incorporation of [γ-^{35} S] GTP, a measure for G protein activation, was quantified by liquid scintillation. The data are expressed as the mean ± SD of triplicates, representing three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared to either no-synaptamide (A) or 0 mM ethanol (B-F) control.

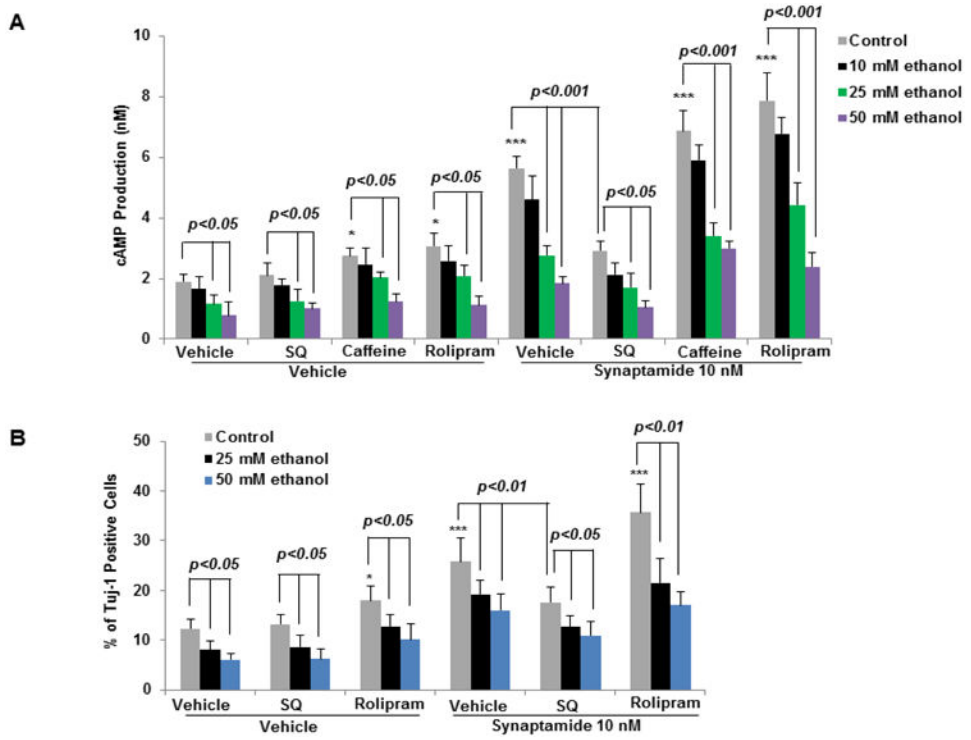

Figure 3. Inhibitor effects on ethanol-inhibited/synaptamide-enhanced cAMP production and neuronal differentiation of NSCs

A-B. Effects of ethanol and synaptamide on cellular cAMP production (A) or neurogenic differentiation (B) persist in the presence of AC or PDE inhibitors. NSCs cultured in the media containing 0-50 mM ethanol for 4 days were treated with the AC inhibitor SQ22536 (100 μM), non-selective (caffeine 10 μM) or selective PDE inhibitor (rolipram 10 μM) for 30 min followed by 10 nM synaptamide for 15 min (A). Subsequently, NSCs were cultured in the corresponding medium for additional 3 days, and Tuj-1-positive neurons (green), and nuclei (blue, DAPI) were visualized by fluorescence microscopy and evaluated using MetaMorph software (B). The data are expressed as the mean ±SD of triplicates, representing three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{\#}p < 0.05$ compared to the no-synaptamide/no-inhibitor DMSO vehicle control.

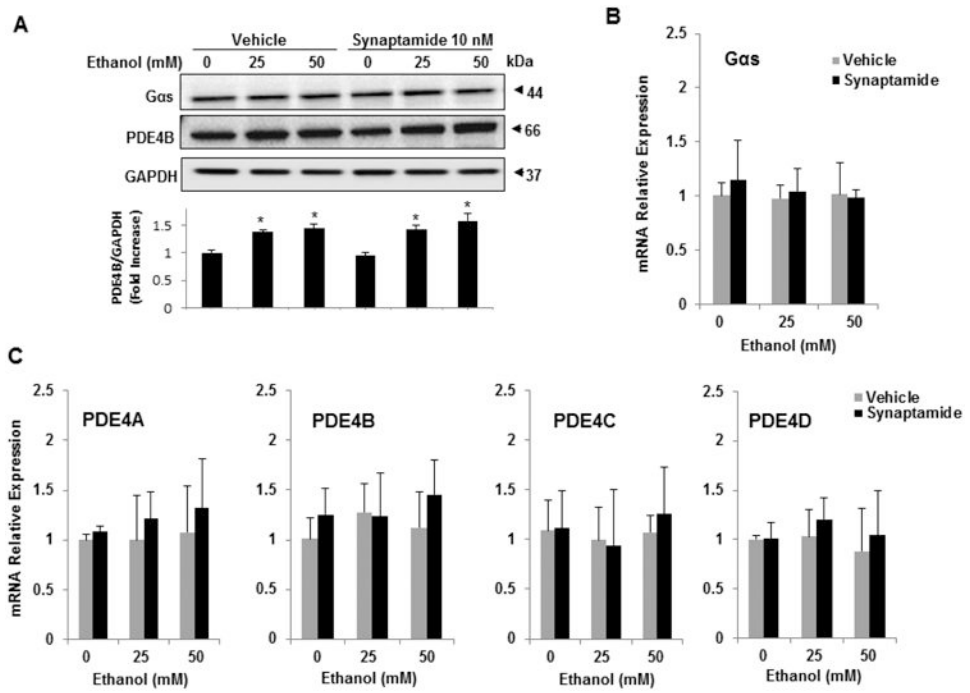

Figure 4. Effects of synaptamide and ethanol on G_S-alpha and PDE4B expression in differentiating NSCs

NSCs were cultured in the media containing ethanol (0-50 mM) for 4 days, and then incubated with synaptamide (10 nM) for additional 24 h in the corresponding media for Western blot analysis (A) or mRNA level of G_S alpha (B) or PDE4 (C) by real-time quantitative RT-PCR. The PDE4 protein level (A) is presented as the fold change of PDE4/GAPDH against no-ethanol/no-synaptamide control. The data are expressed as the mean ± SD of triplicates, representing three independent experiments. *p<0.05.

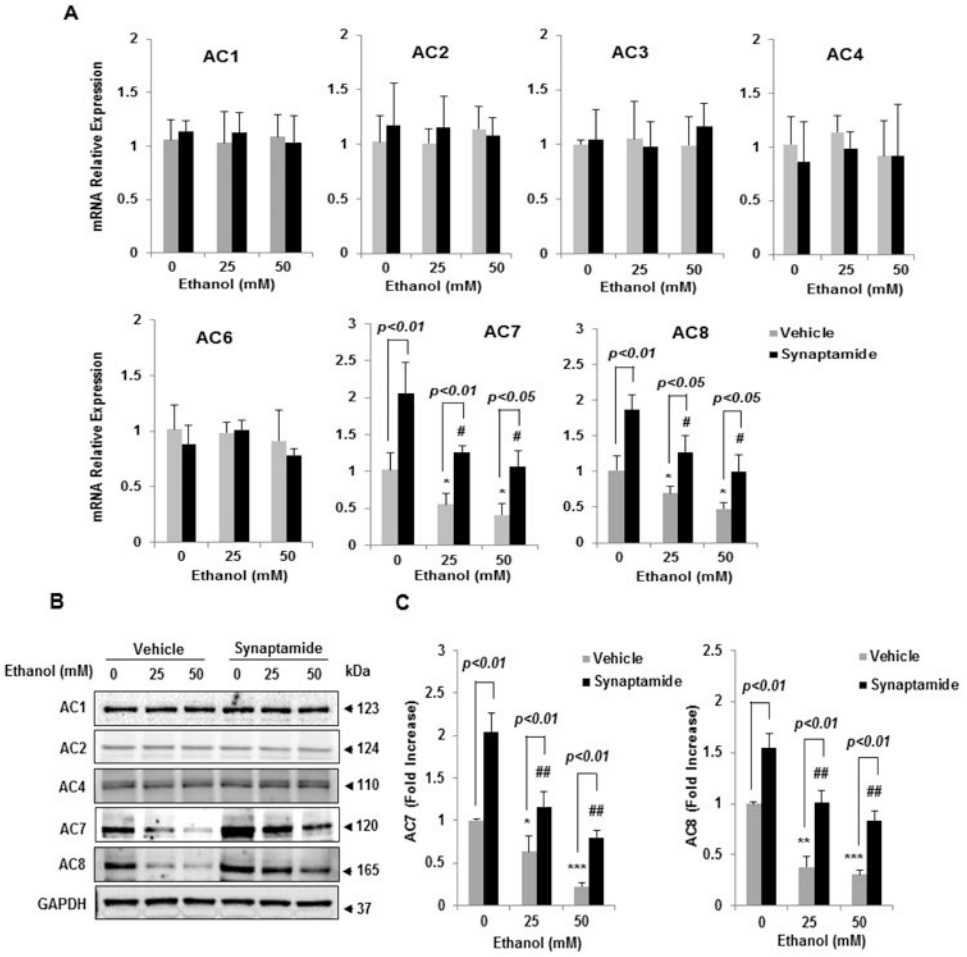

Figure 5. Effect of synaptamide and ethanol on AC expression in differentiating NSCs

A-B. The expression of AC7 and AC8 is specifically and significantly altered by ethanol and synaptamide. NSCs were cultured in the media containing ethanol (0-50 mM) for 4 days, and then incubated with synaptamide (10 nM) for additional 24 h in the corresponding media. The mRNA or protein levels of AC isoforms were analyzed by real-time quantitative RT-PCR (A) or by Western blot analysis (B), respectively. C. Western blot analysis was quantified by densitometry. AC7 and AC8 levels were normalized to the GAPDH intensity. The data are expressed as the mean ± SD of triplicates, representing three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; compared to the corresponding no-ethanol vehicle (*) or synaptamide (#) control.

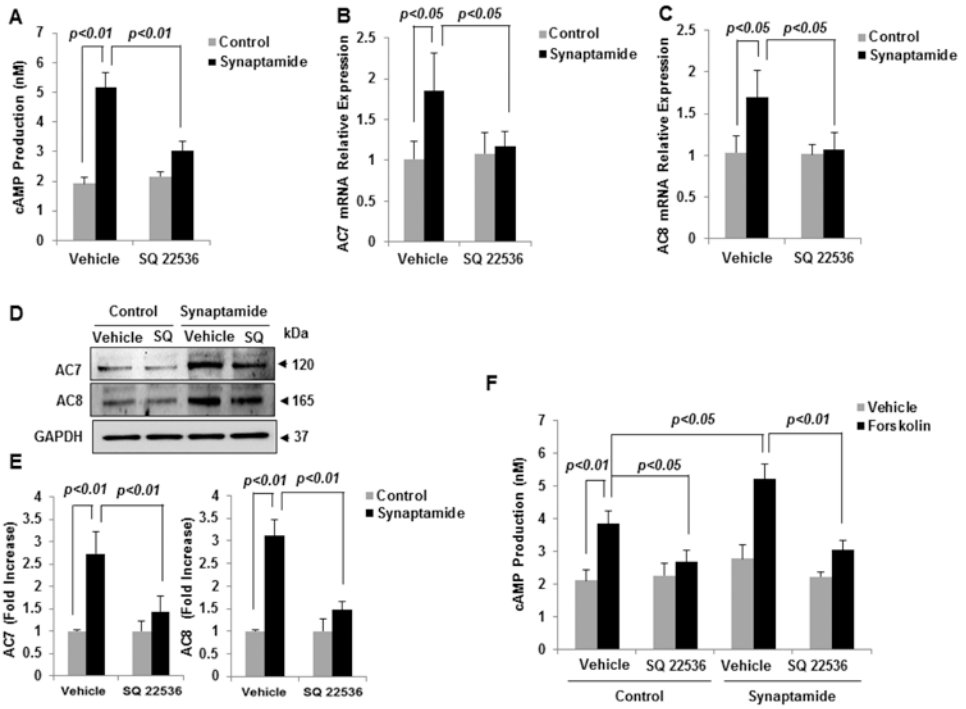

Figure 6. cAMP-dependent induction of functional AC7 and AC8 expression by synaptamide in differentiating NSCs

A-D. Priming DIV4 NSCs with 10 nM synaptamide for 24 h significantly increased the mRNA (B,C) and protein expression (D) of AC7 (B) and AC8 (C), which was prevented by blocking the cAMP increase induced by synaptamide (10 nM, 15 min treatment) using an AC inhibitor SQ22536 (100 μM, 30 min pretreatment) (A). E. Western blot analysis was quantified by densitometry. AC7 and AC8 levels were normalized to the GAPDH signal. F. The AC7 and AC8 isoforms increased by synaptamide are functional as forskolin-stimulated cAMP production in synaptamide-primed NSCs was significantly higher than non-primed or SQ22536-cotreated counterpart. NSCs were treated with 10 nM synaptamide for 24 h in the presence or absence of 100 μM SQ22536, washed, stimulated with 10 μM forskolin or DMSO for 15 minutes and cellular cAMP production was evaluated. The data are expressed as the mean ± SD of triplicates, representing three independent experiments.

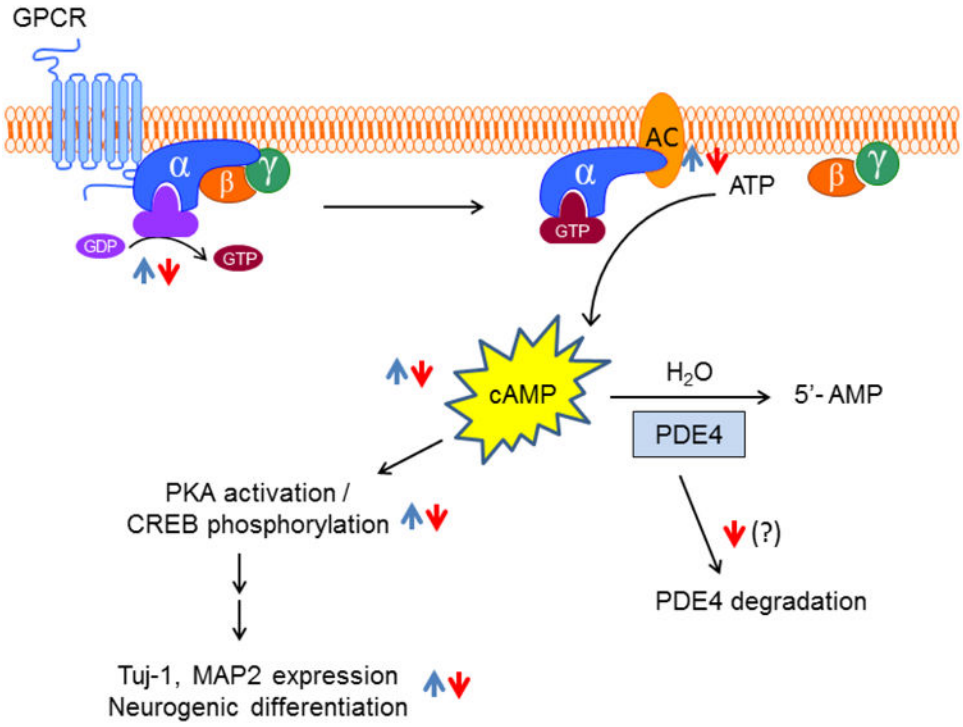

Figure 7. A proposed mechanism for the protective effect of synaptamide on ethanol-mediated inhibition of neurogenic differentiation

Binding of synaptamide to a GPCR triggers the exchange of G-protein bound GDP for GTP. The GTP-bound Gα subunit can then dissociate from the β and γ subunits, and activate AC to produce cAMP which in turn activates PKA/CREB signaling, promoting neurogenic differentiation of NSCs. Cellular cAMP levels are regulated by adenylyl cyclases (AC) and phosphodiesterases (PDE). Chronic ethanol exposure downregulates cAMP by inhibiting GTP/GDP exchange activity and AC7 and AC8 expression, and by increasing cAMP-specific PDE4. By counteracting on G-protein activation and AC expression, synaptamide increases cAMP and ameliorates the attenuating effects of ethanol on neurogenic differentiation of NSCs. Up- or down-regulated effects of ethanol (red) and synaptamide (blue) are indicated by up- or downward arrows, respectively.