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Prenatal ethanol exposure persistently impairs *N*-methyl-D-aspartate receptor-dependent activation of extracellular signal-regulated kinase in the mouse dentate gyrus

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Abstract

The dentate gyrus (DG) is the central input region to the hippocampus and is known to play an important role in learning and memory. Previous studies have shown that prenatal alcohol is associated with hippocampal-dependent learning deficits and a decreased ability to elicit long term potentiation (LTP) in the DG in adult animals. Given that activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling cascade by *N*-methyl-D-aspartate (NMDA) receptors is required for various forms of learning and memory, as well as LTP, in hippocampal regions, including the DG, we hypothesized that fetal alcohol-exposed (FAE) adult animals would have deficits in hippocampal NMDA receptor-dependent ERK1/2 activation. We used immunoblotting and immunohistochemistry techniques to detect NMDA-stimulated ERK1/2 activation in acute hippocampal slices prepared from adult FAE mice. We present the first evidence linking prenatal alcohol exposure to deficits in NMDA receptor-dependent ERK1/2 activation specifically in the DG of adult offspring. This deficit may account for the LTP deficits previously observed in the DG, as well as the life-long cognitive deficits, associated with prenatal alcohol exposure.

Keywords

prenatal alcohol; ERK; NMDA receptor hippocampus; dentate gyrus; learning; memory

Introduction

Fetal Alcohol Syndrome and other prenatal alcohol-related conditions, collectively known as Fetal Alcohol Spectrum Disorder (FASD), affect as many as 40,000 infants born each year in the United States (National Organization on Fetal Alcohol Syndrome, <http://www.nofas.org/faqs.aspx?id=12>). Numerous studies have shown that children exposed to alcohol *in utero* display cognitive deficits, which may not be diagnosed until their educational years, and that these deficits may increase in severity as the individual matures (Streissguth *et al.* 1990; Streissguth *et al.* 1994; Willford *et al.* 2004). Similarly, learning deficits are also observed in animal models of prenatal alcohol exposure (Riley *et al.* 1980, Blanchard *et al.* 1987; Zimmerberg *et al.* 1991, Goodlett and Peterson 1995; Kim *et al.* 1997, Savage *et al.* 2002; Allan *et al.* 2003). Using a two-bottle choice, voluntary drinking paradigm to study the effects of moderate prenatal alcohol exposure, we have previously shown that fetal alcohol-exposed (FAE) mice display hippocampal-dependent

learning deficits (Allan *et al.* 2003). However, the neurochemical abnormalities underlying the learning deficits associated with prenatal alcohol exposure remain unknown.

The discovery that extracellular signal-regulated kinases 1 and 2 (ERK1, 2 - ERK1/2 or ERK), members of the mitogen-activated protein (MAP) kinase family, are widely expressed in post-mitotic neurons in the mammalian nervous system, and are activated in response to excitatory glutamatergic signaling, led to the identification of an important role for ERK1/2 in synaptic plasticity and learning and memory (English and Sweatt 1996; Sweatt 2001; Thomas and Huganir 2004). Activation of the MAPK/ERK cascade is characterized by activation of small G-proteins, such as Rap1 and Ras, which, in turn, activate MAP kinase kinase kinases (MAPKKK), B-Raf and Raf1, which then activate a MAP kinase kinase (MAPKK), such as the MAP kinase/ERK kinase, MEK, by serine/threonine phosphorylation (Sweatt 2001). MEK activates ERK by phosphorylation (phospho-ERK) on both a threonine and a tyrosine residue (Sweatt, 2001). The ERK signaling cascade can be activated by a number of receptors within the hippocampus, including NMDA receptors (Sweatt 2004, see Fig 1). As shown in both hippocampal and cortical cultures, synaptic NMDA receptors are coupled to ERK activation while extrasynaptic NMDA receptors appear to promote ERK inactivation (Ivanov *et al.* 2006; Mulholland *et al.* 2008). ERK activation is coupled to synaptic NMDA receptors via a receptor-mediated increase in intracellular calcium, which leads to the activation of signaling pathways, such as protein kinase A (PKA) and protein kinase C (PKC) and subsequent activation of MEK (see Sweatt 2001, for review).

NMDA receptor-mediated activation of ERK is necessary for hippocampal-dependent learning in rodents, as shown in the hidden platform version of the Morris Water Maze (Blum *et al.* 1999) and a fear conditioning task (Atkins *et al.* 1998). It has been shown *in vitro* that NMDA receptor activation increases ERK phosphorylation in the hippocampus and blocking NMDA receptors with the open channel blocker MK801 or the use of MEK inhibitors prior to training, not only blocks ERK activation, but also prevents hippocampal-dependent learning (Atkins *et al.* 1998; English and Sweatt 1996; Blum *et al.* 1999; Selcher *et al.* 1999). A physiological model of synaptic plasticity that is widely accepted as the cellular mechanism underlying learning and memory is long term potentiation (LTP) (Atkins *et al.* 1998). While it is known that NMDA receptor activation is required for 'classical' LTP (Thomas and Huganir 2004), ERK activation is also essential for LTP induction (English and Sweatt 1997) and has been found to be a requirement even for NMDA receptor-independent forms of LTP (Kanterewicz *et al.* 2000), as well as several forms of LTP within the dentate gyrus (DG) (Coogan *et al.* 1999).

Previous studies have shown that chronic alcohol treatment decreases ERK phosphorylation in adult rat hippocampus (Sanna *et al.* 2002) and acute intraperitoneal injections of ethanol (3.5 g/kg) in postnatal day (PND) 5, PND21 and adult rats also decreases hippocampal phospho-ERK2 (Chandler and Sutton 2005). Thus, alcohol treatment can alter ERK signaling; however, to our knowledge there are no studies to date looking at the effects of prenatal alcohol treatment on ERK activation in adult offspring. Our studies aimed to evaluate ERK activation in a model of prenatal alcohol exposure. Because it is known that NMDA receptor-dependent activation of ERK is necessary for synaptic plasticity, LTP and hippocampal-dependent learning, and because we have shown that fetal alcohol exposed (FAE) adult animals exhibit deficits in hippocampal-dependent learning (Allan *et al.* 2003), we hypothesized that FAE animals would also display deficits in NMDA receptor-dependent activation of ERK within the hippocampal formation. We found a significant deficit in NMDA receptor-dependent ERK activation that is largely localized to the DG of FAE animals. Although most studies in the field of prenatal alcohol research have focused on the CA1 region of the hippocampus (Berman and Hannigan 2000 for review), our results, as

well as other studies, indicate that prenatal alcohol exposure has long lasting effects on the DG (Sutherland *et al.* 1997, see Miki *et al.* 2008 for review).

Materials and methods

Ethanol drinking paradigm

All of the procedures involving animals that were used in the current studies were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee and conformed to NIH guidelines.

C57BL/6J (Jackson Laboratories, Bar Harbor, MA) female mice were individually housed and given standard chow and water *ad libitum*. Prenatal exposure of mice to ethanol was performed using a two-bottle choice paradigm with water and 0.066% (w/v) saccharin-sweetened ethanol solutions, essentially as described previously (Allan *et al.* 2003). Briefly, ethanol concentrations were increased in a stepwise fashion from 0 to 2.5%, then two days later to 5%. After the female was consistently drinking 5% ethanol, a male was introduced and breeder chow and water *ad libitum* were provided. Once a female was determined to be pregnant, the male was removed, nesting material was added to the cage and the female continued to have access to 5% ethanol throughout pregnancy. Control groups were also given the two-bottle choice paradigm with free access to 0.066% (w/v) saccharin-sweetened water, instead of saccharin-sweetened ethanol solution. Maternal ethanol consumption was measured every other day, after the male was removed from the cage, until the pups were born. The g ethanol consumed / kg body weight / day were calculated for each litter based on consumption of the mother during pregnancy. As noted in (Allan *et al.* 2003), total maternal fluid consumption during the two-bottle choice paradigm did not differ between ethanol- and saccharin- drinking mothers and both groups preferred the saccharin sweetened solution (either ethanol or water) over regular tap water. Once the pups were born, ethanol and saccharin concentrations were reduced to zero within 4 days of parturition in a stepwise fashion. Pups were weaned between 21-23 days and maintained in same sex, littermate cages with free access to food and water.

Hippocampal slice preparation and *in vitro* stimulation

Saccharin control (Sacc) and FAE offspring (2-5 months of age) were anesthetized with ketamine (250 mg / kg body weight) and decapitated. Brains were removed and placed in ice-cold cutting solution containing (in mM): 3 KCl, 1.25 NaH₂PO₄, 12 MgSO₄, 26 NaHCO₃, 0.2 CaCl₂, 10 glucose, 220 sucrose, and 0.43 ketamine. Coronal sections (300 μ m) were cut with a Vibratome (Technical Products, St. Louis, MO) and the slices were transferred to artificial cerebral spinal fluid (ACSF) containing (in mM): 126 NaCl, 2 KCl, 1.25 NaH₂PO₄, 1 MgSO₄, 26 NaHCO₃, 2 CaCl₂, and 10 glucose, equilibrated with 95% O₂ / 5% CO₂, at 37 °C for 45 min. Slices were then incubated at room temperature for an additional 45 min. For NMDA stimulations, the slices were washed 3X (5 min each) in magnesium-free ACSF equilibrated with 95% O₂ / 5% CO₂ after recovery.

For all experiments, the whole hippocampus was dissected and treated with or without 50 μ M forskolin (FSK) (Calbiochem, San Diego, CA) for 10 min, 3 μ M Phorbol 12,13 diacetate (PDA) (Sigma, St. Louis, MO) for 10 min, or 25/50/100 μ M NMDA (Sigma) for 3 min at room temp. Unstimulated (control) slices were incubated in ACSF (magnesium-free for NMDA incubations) for the corresponding time. For MEK inhibitor experiments, slices were incubated in 20 μ M U0126 (Calbiochem) for 30 min at room temperature under constant 95% O₂ / 5% CO₂ prior to treatment with or without NMDA. NMDA inhibitor experiments were performed using 100 μ M 2-amino-5-phosphonovaleric acid (APV) and 50 μ M MK801 (Calbiochem); both inhibitors were present in the dissecting magnesium-free

wash, as well as during the incubation with or without NMDA. The reaction was terminated by addition of ice-cold homogenization buffer (HB) containing (in mM) 20 Tris, pH 7.4, 1.0 EDTA, 320 sucrose, 20 sodium pyrophosphate, 10 sodium fluoride, 20 β -glycerophosphate and 0.2 sodium orthovanadate, and protease inhibitor cocktail (cat# P8340 Sigma), pH 7.4 and sonication on ice for 15 seconds. Samples were then frozen in liquid nitrogen and stored at -80 °C. Total protein concentrations were determined using the Bio-Rad (Hercules, CA) Protein Assay (Bradford method) with Bovine Albumin Serum (BSA) as a standard.

Immunoblotting for ERK in samples from hippocampal slices

Anti-ERK2 and anti-phosphoERK2 (pERK2) immunoreactivities were determined from *in vitro*-stimulated hippocampal slice preparations (see above). Samples were thawed on ice, NuPAGE 4X sample buffer (Invitrogen Corporation, Carlsbad, CA) was added and samples were heated at 70 °C for 10 min. Samples were electrophoresed using NuPage 12% Bis-Tris pre-cast gels (Invitrogen) and transferred to 0.45 μ m nitrocellulose membranes (Invitrogen). Membranes were blocked in 0.25% I-block (Tropix, Applied Biosystems, Foster City, CA) in Tris-buffered saline (TBS) containing (in mM): 20 Tris, 150 NaCl and 0.05% (v/v) Tween-20, pH 7.5 and probed with a rabbit polyclonal anti-pERK1/2 antibody at 1:3000 (Cell Signaling, Danvers, MA) followed by goat anti-rabbit secondary antibody (Pierce, Rockford, IL). The immunoreactive proteins were detected by using Pierce Supersignal West Pico Chemiluminescence. Blots were then stripped using Restore Western Blot Stripping Buffer (Pierce) and re-probed with rabbit polyclonal anti-total ERK1/2 antibody at 1:3000 (Cell Signaling) followed by goat anti-rabbit secondary antibody (Pierce).

Western Blot Data Analysis

ERK2 activation was assessed using immunoblotting techniques was determined by calculating the ratio of anit-pERK2 immunoreactivity relative to the amount of anti-total ERK2 immunoreactivity present in each of the slice samples. Slice samples were not fractionated prior to immunoblotting, thus they represent ERK activation throughout all cellular regions. In order to compare across multiple gels, optical densities of all bands were normalized to immunoreactivities present in a 1000 xg postnuclear hippocampal standard preparation run on every gel. Data were graphed and analyzed using GraphPad 3.0/4.0 and SPSS 15.0 program using ANOVA and Tukey's post hoc test. Standard unpaired t-test was also used, when applicable.

Immunohistochemistry of re-sectioned hippocampal slices

Hippocampal slices were prepared as described above. Following treatment with or without 100 μ M NMDA, slices from Sacc and FAE animals were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), pH 7.4. Slices were then cryoprotected for at least 24hrs in 30% sucrose in PBS before being frozen in isopentane using Tissue Tek cryomolds and Optimal Cutting Temperature (OCT) compound (VWR Scientific, West Chester, PA) and stored at -80°C prior to resectioning. 16 μ m cryostat sections were then prepared, thaw-mounted onto Superfrost-Plus microscope slides (VWR Scientific) and stored at -80°C.

Air-dried slides were labeled with Super PAP Pen HT™ Slide marker (Research Products International, Mount Prospect, IL) and blocked with 5% normal donkey serum (Sigma) in PBS-0.3% Triton X-100 for 1 hr at room temperature. Sections were then incubated overnight at 4°C with rabbit monoclonal anti-pERK1/2 (Cell-Signaling) at 1:100 or rabbit polyclonal anti-total ERK1/2 (Cell Signaling) at 1:25. After primary antibody incubation, slides were washed three times (10 min each with PBS) and incubated for 2 hrs at room temperature in the dark with Alexa Fluor 555 donkey anti-rabbit IgG (Invitrogen) at 1:1000. After removal of the secondary antibody, sections were washed as previously described and

counterstained with 4', 6-diamidino-2-phenylindole, dilactate (DAPI) (Invitrogen) in PBS at 1:1000 for 15 min and washed again. 1-2 drops of Vectashield mounting medium (Vector Laboratories, Burlingame, CA) was added to the section to prevent photobleaching before coverslipping. Immunostained sections were viewed and imaged using a Zeiss META LSM 510 confocal microscope system (Thornwood, NY). A Laser Diode laser with 405 nm excitation was used to excite DAPI and a Helium Neon (HeNe1) laser with 543 nm excitation was used to excite the Alexa Fluor 555 which emits at 565 nm. Both 4X and 10X objectives were used with numerical apertures of 0.13 and 0.3, respectively.

Quantification of Confocal Images

Images were quantified using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2008). Briefly, mean intensities were calculated for pERK1/2, total ERK1/2 and secondary alone (background) from images collected using a 10X objective. Background mean intensities were subtracted from pERK1/2 and total ERK1/2 values and a ratio of pERK1/2 to total ERK1/2 was calculated for each hippocampal region in order to assess ERK1/2 activation. The area of ERK1/2 activation was calculated using Image J software on images obtained using a 4X objective. Briefly, the area of ERK1/2 activation, visualized by the increase in Alexa Fluor 555 staining in the pERK1/2 images, was outlined and measured for the CA1 regions of both Sacc and FAE slices treated with NMDA using the free-hand selection tool on Image J. All image analysis was done blind.

Results

Fetal Alcohol Exposure paradigm

The voluntary two-bottle choice fetal alcohol exposure paradigm was conducted as previously described (Allan *et al.* 2003; Caldwell *et al.* 2008) using C57BL/6J mice. Previous studies using this model (Caldwell *et al.* 2008) showed that the maternal blood ethanol concentrations (BECs) of C57BL/6J dams consuming an average of 8.0 g ethanol / kg body weight / day throughout gestation, were 78 mg / dL (n=7). The animals used in the following experiments were from litters born to dams having an average maternal consumption of 9.49 (± 2.44) g ethanol / kg body weight / day. As in our previous report employing C57BL/6J mice and this model (Caldwell *et al.* 2008), there were no differences between Sacc and FAE maternal weights prior to beginning the drinking paradigm or one week after birth; there were also no differences in litter sizes. With this model, we previously showed that alcohol drinking during pregnancy does not cause significant differences in maternal care (Allan *et al.* 2003) and we noted no differences in the present cohorts of mice.

Western immunoblotting analysis of ERK2 in hippocampal slices

NMDA-dependent ERK activation—NMDA is known to activate ERK1/2 in hippocampal slices *in vitro* (Banko *et al.* 2004; English and Sweatt 1996). We measured the NMDA receptor-dependent phosphorylation of ERK2 in acute hippocampal slices prepared from adult FAE and Sacc animals by immunoblotting techniques. Although the anti-phospho and anti-total ERK1/2 antibodies used in these studies recognize both ERK1 and ERK2, ERK1 was not analyzed because it was detected at much lower levels than ERK2 and, thus, ERK1 bands were often too faint and inconsistent to accurately analyze. Additionally, we chose from the start of these studies to focus on ERK2 since previous studies by Selcher *et al.* (2001) had revealed that mice lacking ERK1 have unimpaired memory formation, which suggests that, at least in the processes underlying learning and memory, ERK2 may be able to compensate for reduced ERK1 levels.

In order to optimize the concentration of NMDA used in our experiments, hippocampal slices from FAE and Sacc control animals were treated with 0, 25, 50 or 100 μ M NMDA for 3 min and the levels of pERK2 and total ERK2 were assessed by immunoblotting techniques (Fig 2A). Fig. 2B shows the effects of increasing NMDA concentrations on anti-pERK2 immunoreactivity in Sacc and FAE hippocampal slices. Two-way ANOVA of the pERK2 data demonstrated a significant effect of prenatal diet [$F(1,56) = 6.23$, $p < 0.02$] and an effect of NMDA treatment that approached significance [$F(3,56) = 2.47$, $p = 0.071$]. As expected, there was no effect of NMDA treatment on total ERK2 levels in slices prepared from Sacc or FAE mice (Fig. 2C) and total ERK2 levels were not significantly different between the groups. In Fig. 2D, ERK2 activation was assessed by expressing the ERK2 data as a ratio of pERK2 to total ERK2. Two-way ANOVA revealed a significant effect of prenatal diet [$F(1,56) = 7.19$, $p = 0.010$] and a significant effect of NMDA treatment [$F(3,56) = 7.87$, $p < 0.01$]; however, there was no interaction [$F(3,56) = 1.79$, $p = 0.1596$]. Tukey's post hoc analysis revealed that both 50 and 100 μ M NMDA produced a significant increase in ERK2 activation above basal levels (0 NMDA) in the Sacc group, while none of the concentrations of NMDA elicited significant ERK2 activation in the FAE group.

In a separate group of animals, we stimulated hippocampal slices with 100 μ M NMDA in the presence and absence of two NMDA receptor inhibitors (50 μ M MK801 and 100 μ M APV) or a MEK inhibitor (20 μ M U0126). Representative Western blots are shown in Fig 3A, B. As found in the study presented in Figure 2, NMDA treatment did not affect total ERK2 levels and there was no significant difference in total ERK2 between the groups (data not shown). Similarly, pretreatment in U0126 and MK801 / APV did not affect total ERK2 levels in either group (data not shown). Two-way ANOVA of the calculated ERK2 activation data (ratio of anti-pERK2 immunoreactivity to the anti-total ERK2 immunoreactivity) shown in Fig 3C revealed a significant effect of treatment [$F(5,52) = 42.2$, $p < 0.01$]. Post hoc analysis showed that, similar to the study presented in Figure 2, 100 μ M NMDA significantly activated ERK2 in Sacc, but not FAE, slices (Fig. 3C). Pretreatment with NMDA inhibitors MK801 and APV significantly blocked the NMDA-induced ERK2 activation in Sacc slices, but did not produce a significant effect in FAE slices. Both basal and NMDA-stimulated pERK2 levels were greatly reduced while total ERK2 levels were not altered in slices pretreated with the MEK inhibitor U0126 (Fig 3A). Consequently, ERK2 activation was significantly decreased by pretreatment with U0126 compared to untreated control slices (Fig 3C).

Downstream signaling pathways coupling NMDA receptor activation to ERK are intact—In order to determine whether the observed deficit in ERK2 activation in FAE slices was dependent on engagement of the NMDA receptor, we bypassed the NMDA receptor and activated ERK2 using phorbol 12,13 diacetate (PDA), which activates PKC, and forskolin (FSK), which is known to activate PKA via stimulation of adenylyl cyclase. For simplicity, we looked at activation of either PKA or PKC independently. Both PDA and FSK have previously been shown to induce ERK2 phosphorylation in hippocampal slices (Banko *et al.*, 2004; English and Sweatt, 1996). We found that PDA stimulation of hippocampal slices produced significant activation of ERK2 in both Sacc and FAE slices: two-way ANOVA of the data revealed a significant effect of PDA treatment [$F(1,20) = 25.11$, $p < 0.01$] (Fig. 4A, B). There was no difference in basal or PDA-stimulated ERK2 activation between the groups. Similarly, we found that FSK treatment of hippocampal slices also produced significant increases in ERK2 phosphorylation in both groups and two-way ANOVA of the data revealed a significant effect of FSK treatment [$F(1,28) = 10.28$, $p < 0.01$] (Fig. 4C, D). Neither PDA nor FSK had a significant effect on total ERK2 (Figs 4A, C and data not shown).

Immunohistochemistry of NMDA receptor-dependent ERK1/2 activation in hippocampal slices

We explored the NMDA receptor-dependent ERK2 deficit more closely using immunohistochemical techniques. Acute hippocampal slices from Sacc and FAE animals were treated with 100 μ M NMDA for 3 minutes, then fixed, frozen, re-sectioned and incubated with anti-ERK1/2 antibodies in order to determine whether the prenatal alcohol-induced deficits in ERK activation were regional or global throughout the hippocampus. Although activation was slightly variable, both Sacc and FAE slices displayed increased ERK1/2 activation following NMDA treatment (Fig 5, Fig 6). Figure 5A shows representative 4X images from Sacc and FAE hippocampal slices treated without or with 100 μ M NMDA for 3 min (-NMDA, +NMDA). Hippocampal regions CA1 and CA3, as well as the DG, are labeled on the images. In both Sacc and FAE slices the most prominent ERK1/2 activation was in the CA1 region. Initial observations from the images suggested that FAE slices may have a smaller area of ERK1/2 activation in the CA1 region (Fig 5A). However, analysis of the area of ERK1/2 activation in multiple animals showed that the observed difference was not significant (Fig 5B).

Higher magnification (10X) of the slides revealed that NMDA-dependent activation of ERK1/2 was detectable in the hippocampal CA1 and DG in Sacc control slices (Fig. 6A) but only in the CA1 region in FAE slices (Fig. 6B). In contrast, NMDA-stimulated activation of ERK1/2 was not observed in the hippocampal CA3 region in either Sacc control or FAE slices. ERK1/2 activation in the CA1 appeared to occur primarily in pyramidal cell dendrites located in the stratum radiatum (s.r.), with less activation in the stratum oriens (s.o.) and essentially no activation in the stratum pyramidale (s.p.), in both Sacc and FAE groups (Fig 6A,B). As recorded in the immunoblotting studies (Fig. 2), NMDA treatment did not affect total ERK1/2 levels in any region (data not shown). The mean intensity of the anti-pERK1/2 and anti-total ERK1/2 signals were quantified and used to calculate the pERK1/2 to total ERK1/2 ratio, an indirect measure of ERK1/2 activation. Two-way ANOVA of ERK1/2 activation data revealed a significant effect of NMDA treatment in the CA1 $F(1,18) = 34.07$, $p < 0.0001$, with post hoc analysis revealing a significant increase in both Sacc ($***p < 0.0001$) and FAE ($**p < 0.01$) groups upon NMDA stimulation (Fig 7A). Activation of ERK1/2 in the DG appeared to occur along the blades of the DG in Sacc control slices, as indicated by white arrows in Fig 6A. FAE slices showed little to no ERK1/2 activation following NMDA treatment in the DG (Fig 6B). Two-way ANOVA of ERK1/2 activation data for the DG revealed a significant effect of NMDA treatment $F(1,18) = 7.264$, $p < 0.05$ and a significant effect of prenatal diet $F(1,18) = 5.216$, $p < 0.05$, while the interaction between these two factors did not quite achieve significance $F(1,18) = 3.727$, $p = 0.0695$ (Fig 7B). Post hoc analysis revealed a significant increase in ERK1/2 activation following NMDA treatment in the DG of Sacc slices ($*p < 0.05$), but not in FAE slices (Fig 7B).

Discussion

To the best of our knowledge, this is the first study to demonstrate that prenatal alcohol exposure disrupts hippocampal NMDA receptor-dependent regulation of ERK1/2 in adult offspring. Furthermore, within the adult mouse hippocampal formation, we localized this deficit specifically to the DG. Other studies have also reported that the DG is particularly sensitive to the effects of prenatal alcohol exposure (Sutherland *et al.* 1997; Miki *et al.* 2008). In view of these findings, we propose that the observed deficit in NMDA receptor-dependent regulation of ERK1/2 in the DG underlies the LTP deficits previously seen by Sutherland and colleagues (1997) and may, in part, account for the cognitive impairments commonly associated with prenatal alcohol exposure. The deficit appears to lie upstream of PKC and PKA signaling to ERK; for example, at the level of the NMDA receptor. Possible alterations could include: differential subunit expression, differential subcellular targeting of

receptor subunits, impaired functioning of the receptor-ion channel, or the interaction of the receptor with signal transduction systems that couple it to ERK1/2. Other potential alterations include defective Ca^{2+} buffering, decreased availability of Ca^{2+} cofactors, such as calmodulin, or modifications in other signaling pathways that contribute to the activation of the ERK signaling cascade, such as calcium/calmodulin-dependent kinase CaMKII or Ras-specific GDP/GTP exchange factor, RasGRF1 (see below).

FAE slices exhibited reduced ERK2 activation at all concentrations of NMDA used

It was important to optimize the concentration of NMDA used in our experiments, as exposure of neuronal cultures to relatively high concentrations of NMDA (70–100 μM) had been previously reported to cause inactivation of ERK2 via binding to and activating extrasynaptic NMDA receptors (Chandler *et al.* 2001; Ivanov *et al.* 2006; Kim *et al.* 2005). In contrast to these reports, we did not find measurable ERK2 inactivation in either Sacc control or FAE adult mouse hippocampal slice preparation treated with 100 μM NMDA. It is possible that these differing results may be due to the use of different preparations (cultures vs. slices) or differences in experimental protocols (e.g., time of exposure). Although Sacc and FAE slices displayed NMDA concentration-dependent ERK2 activation, FAE slices appeared to have a blunted response at all concentrations of NMDA used. These data demonstrate that prenatal alcohol exposure causes damage to NMDA receptor-dependent ERK2 activation in the hippocampus that persists into adulthood. It is worth noting that we analyzed ERK2 activation in whole hippocampal slice preparations that had been sonicated following NMDA stimulation. Thus, these studies measured ERK2 activation throughout the cell and did not distinguish between nuclear or cytosolic pools of ERK. Nuclear and cytosolic pools of ERK1/2 have been proposed to regulate differing cellular responses (see Sweatt 2001 for review). Future studies will aim to address the effects of prenatal ethanol exposure on these different pools of cellular ERK.

NMDA-stimulated ERK activation is dependent on both MEK and NMDA receptor activation

Prenatal alcohol-induced deficits in NMDA receptor-dependent ERK2 activation were replicated in the inhibitor experiments. Pretreatment with NMDA receptor inhibitors MK801 and APV did not affect basal levels of ERK2 activation, but it did prevent an increase in ERK2 activation following NMDA stimulation. This demonstrates that NMDA receptors do not play an important role in the control of basal levels of phosphorylated ERK2; however, ERK2 activation following NMDA stimulation does require NMDA receptor activation in adult mouse hippocampal slices. U0126, an inhibitor of MEK, blunted basal levels of ERK2 activation without affecting total levels of ERK2 in both groups, thus showing that, while basal ERK activation is not dependent on NMDA receptor activation, it is dependent on MEK. Pre-incubation with U0126 also prevented NMDA-induced ERK activation in slices thus demonstrating that both basal and NMDA-dependent activation of ERK2 requires MEK activation.

PKA- and PKC- dependent ERK activation are unaffected by prenatal alcohol exposure

Multiple signaling pathways, including those in which PKA and PKC are critical components, lead to ERK activation following NMDA receptor agonist binding (Sweatt 2001, for review). PKA and PKC activate ERK via Rap1 - B-Raf - MEK and Ras — Raf1 - MEK signaling, respectively (Grewal *et al.* 1999; Sweatt 2001). Research has shown that, although both PKA and PKC can activate ERK, PKA inhibits the Ras/Raf1 pathway by preventing Raf1 activation and attenuating its activity (Sweatt 2001); thus PKA may hinder PKC-mediated activation of ERK. Additionally, studies show that cAMP can positively couple to ERK activation via Rap-1 and B-Raf without involvement of PKA (Vossler *et al.* 1997). However, despite the complexity of PKA- and PKC- mediated ERK activation, both signaling molecules mediate ERK1/2 activation via activation of MEK. We examined ERK2

activation via these pathways and found them to be intact in FAE hippocampal slices. These results indicate that there is nothing inherently defective about ERK2 activation in FAE hippocampal slices, nor in the PKA and PKC signaling pathways leading to ERK2 activation downstream of the NMDA receptor, indicating that the prenatal alcohol-induced deficit lies upstream of these kinases. While another kinase, CaMKII, has been implicated in NMDA receptor-mediated ERK activation (Grewal *et al.* 1999; Sweatt 2001; Illario *et al.* 2003; Thomas and Huganir 2004), conflicting reports on the mechanism by which CaMKII can affect ERK activation (Chen *et al.* 1998; Oh *et al.* 2004), as well as reports suggesting it may have a more prominent role in regulating other MAPKs as opposed to ERK (Krapivinsky *et al.* 2004), deterred us from evaluating CaMKII in the current study. Additional regulators of ERK activity include dualspecificity phosphatases, serine/threonine phosphatases and tyrosine phosphatases (Paul *et al.* 2003), that inactivate this signaling pathway. Striatal-enriched protein tyrosine phosphatase (STEP) has been suggested to play a role in extrasynaptic ERK inactivation (Hardingham 2006). STEP has been shown to be activated by NMDA receptor-mediated calcium influx and limits the duration of ERK activity in neuronal cultures (Paul *et al.* 2003). Because phosphatases play an important role in the mediation of ERK activity, future studies will evaluate if the activity of these phosphatases is altered by prenatal alcohol exposure.

Prenatal alcohol exposure is associated with impaired NMDA-induced ERK1/2 activation in the DG

We observed that the majority of NMDA receptor-mediated ERK1/2 activation in hippocampal slices was localized to the CA1 region of the hippocampus, specifically in the pyramidal cell dendrites within the stratum radiatum. Consistent with these studies, Banko *et al.* (2004) reported significant ERK2 activation was observed in microdissected CA1 region after 100 μ M NMDA stimulation of hippocampal slices for 3 min. There was no significant difference in ERK1/2 activation following NMDA stimulation in the CA1 of Sacc and FAE slices. In contrast, NMDA-dependent activation of ERK1/2 was not observed in the hippocampal CA3 region of either group. Studies by Coultrap and colleagues show that there is significantly less NMDA receptor subunit NR1 expression in the CA3 compared to CA1 or DG (Coultrap *et al.* 2005), thus supporting our findings that NMDA stimulation did not produce ERK1/2 activation in CA3. Most significantly, NMDA receptor-mediated ERK1/2 activation was observed in the DG of control slices, but not FAE slices. Both the CA1 and DG have been shown to be important for learning and memory (Dillon *et al.* 2008; Hernandez-Rabaza *et al.* 2008); the DG is the entry point for input into the hippocampus and DG lesions have been shown to dramatically affect spatial memory (Jeltsch *et al.* 2001). Studies have shown that knock out of the NMDA receptor subunit NR1, which is the mandatory subunit for a functional receptor, in the DG severely impairs LTP in perforant path inputs to the DG (McHugh *et al.* 2007; Niewoehner *et al.* 2007), as well as impairs spatial working memory in a radial arm maze task (Niewoehner *et al.* 2007). These studies solidify the role of NMDA receptors and their subsequent signaling in synaptic plasticity. In addition to the studies showing that ERK activation is required for LTP in the DG (Coogan *et al.* 1999), our findings of significantly impaired NMDA receptor-dependent activation of ERK in the DG of FAE mice are consistent with the previously reported DG LTP and spatial memory deficits observed in prenatal alcohol-exposed adult animals (Sutherland *et al.* 1997; Savage *et al.* 2002).

It has been shown that prenatal alcohol exposure is associated with decreased volume of the DG, as well as aberrant distribution of granule cell axon terminals (see Miki *et al.* 2008 for review), and can decrease LTP in perforant path-dentate synapses (Sutherland *et al.* 1997). Furthermore, studies by Savage and colleagues found that prenatal alcohol induced significant reductions in NMDA-sensitive ^3H -glutamate binding in the DG (Savage *et al.*

1991) and radial arm maze learning and memory (Reyes *et al.* 1989) in adult rats. While no structural abnormalities within the DG were apparent in our studies of FAE slices, we show evidence that prenatal alcohol induces abnormalities in NMDA receptor signaling to the ERK1/2 cascade. The fact that these animals were only exposed to alcohol *in utero* and were not exposed to alcohol during postnatal, adolescent or adult periods suggests that this signaling deficit is a developmental deficit occurring during the first and second trimester equivalents and that it is localized to the DG.

Although our immunoblotting data suggest that prenatal alcohol induces small effects on NMDA receptor-dependent ERK activation, the immunohistochemistry data revealed that this deficit is of significant magnitude in the DG. The effect may have appeared to be small in the immunoblotting studies due to its dilution with the incorporation of CA3, which showed basal ERK1/2 phosphorylation, and CA1, which showed the majority of NMDA-induced ERK1/2 activation. Taken together, our immunoblotting data and immunohistochemistry data support the conclusion that prenatal alcohol exposure is associated with deficits in NMDA receptor-dependent ERK activation.

Possible mechanisms for decreased NMDA receptor-dependent ERK activation in FAE animals

While this is the first report of prenatal alcohol exposure having effects on the activation of the NMDA receptor-dependent ERK signaling pathway that persist into adulthood, we recognize that this is just one part of a complex mechanism leading to the cognitive deficits associated with fetal alcohol exposure. Research suggests that prenatal alcohol exposure is associated with alterations in several properties of NMDA receptors, including receptor subunit composition (Hughes *et al.* 1998; Nixon *et al.* 2004; Toso *et al.* 2005; Honse *et al.* 2003; Samudio-Ruiz *et al.* in preparation, but see Costa *et al.* 2000), ³H-MK801 binding (Diaz-Granados *et al.* 1997; but see Morrisett *et al.* 1992), and receptor function (Morrisett *et al.* 1989; Gruol *et al.* 1998; Spuhler-Phillips *et al.* 1997). With the exception of the work by Sutherland and colleagues showing decreased LTP in perforant path-dentate synapses (Sutherland *et al.* 1997), prenatal alcohol-induced alterations in NMDA receptor function have not been fully evaluated in DG of adult offspring. Future studies should evaluate the effects of prenatal alcohol exposure on NMDA receptor function, subunit composition, subcellular targeting of receptors and the interaction of the receptor with intracellular signaling molecules such as the Ras activator that leads to ERK activation, RasGRF1 (see Krapivinsky *et al.* 2003 for review) in the DG of adult offspring.

Conclusions

In summary, we found that FAE mice have decreased hippocampal NMDA receptor-dependent ERK activation that was not due to inherent deficiencies in PKA (Rap1 - B-Raf — MEK) or PKC (Ras — Raf1 — MEK) signaling. Immunohistochemical studies demonstrated that the deficit was localized to the DG, which is known to be important for spatial learning and thought to be essential for memory storage (Niewoehner *et al.* 2007; Miki *et al.* 2008). This observation, combined with studies showing that prenatal alcohol exposure affects development of the DG (Miki *et al.* 2008) and is associated with deficits in long LTP in perforant path-dentate synapses (Sutherland *et al.* 1997), suggests that prenatal alcohol-induced deficits in NMDA receptor-dependent ERK activation within the DG of adult animals may account for some of the life-long learning and memory deficits associated with prenatal alcohol exposure. The current literature and the studies presented here suggest that additional research on the effects of prenatal alcohol on the development and functioning of this key component of the hippocampal formation is warranted.

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Fig. 1.

NMDA receptor-dependent activation of ERK1/2 mediated by PKA and PKC. Synaptic NMDA receptors can induce ERK1/2 activation via increases in intracellular calcium and subsequent activation of calcium/calmodulin dependent adenylyl cyclase (AC) which produces cAMP and activates PKA, or intracellular calcium can activate calcium-dependent protein kinase C (PKC). PKA activates ERK1/2 via Rap1-B-Raf-MEK and PKC activates ERK1/2 via Ras-Raf1-MEK (see text). PKA can inhibit Raf-1 and potentially hinder PKC-mediated ERK1/2 activation. Extrasynaptic NMDA receptors do not activate ERK1/2; rather, they inhibit ERK1/2 via activation of calcium-dependent phosphatases, possibly striatal-enriched protein tyrosine phosphatase (STEP).

Fig. 2. NMDA-dependent activation of ERK2 in saccharin control (Sacc) and fetal alcohol-exposed (FAE) adult offspring hippocampal slices, $n=8$. (A) Representative phospho-ERK1/2 (pERK1/2; top) and total ERK1/2 (bottom) Western blots for both Sacc and FAE hippocampal slices treated with 0, 25, 50 and 100 μ M NMDA. (B) Levels of pERK2 in Sacc and FAE samples at various concentrations of NMDA. Two-way ANOVA of the pERK2 data showed a significant effect of prenatal diet ($p<0.02$). (C) Total ERK2 levels in Sacc and FAE hippocampal slices with increasing NMDA concentration. There were no significant differences between groups or with NMDA treatment on total ERK2 levels. (D) ERK2 activation state expressed as a ratio of pERK2 to total ERK2 in Sacc and FAE hippocampal slices treated with 0, 25, 50 and 100 μ M NMDA. Two-way ANOVA revealed significant main effects of prenatal diet ($p=0.010$) and NMDA ($p<0.01$), but no significant interaction. All levels of pERK2 and total ERK2 are expressed as optical densities normalized to a hippocampal standard preparation loaded in all gels in order to compare across gels. Sacc and FAE samples were run on the same gel in order to compare between the groups and

blots were done in duplicate. Total ERK2 immunoreactivity was evaluated on all blots and used to generate pERK2/total ERK2 ratio for each sample.

Fig. 3.

ERK2 activation in saccharin control (Sacc) and fetal alcohol-exposed (FAE) adult hippocampal slices treated with 100 μ M NMDA in the presence and absence of MEK inhibitor U0126 or NMDA receptor inhibitors MK801 & APV. (A) Representative phospho-ERK1/2 (pERK1/2; top) and total ERK1/2 (bottom) Western blots for both Sacc and FAE hippocampal slices treated with 100 μ M NMDA in presence and absence of MEK inhibitor 20 μ M U0126. (B) Representative pERK1/2 (top) and total ERK1/2 (bottom) Western blots for both Sacc and FAE hippocampal slices treated with 100 μ M NMDA in presence and absence of NMDA inhibitors 100 μ M APV & 50 μ M MK801. (C) ERK2 activation data expressed as a ratio of pERK2 to total ERK2 for Sacc and FAE slices treated, or untreated, with 100 μ M NMDA (n=8) and pretreated, or not, with 50 μ M MK801 / 100 μ M APV (n=4), or with 20 μ M U0126 (n=4). Two-way ANOVA revealed a significant effect of treatment ($p<0.01$). Post hoc analysis showed a significant increase in ERK2 activation in +NMDA compared to -NMDA Sacc slices (** $p<0.01$) and a significant difference between +NMDA versus +NMDA + MK801 / APV data in Sacc slices (* $p<0.05$). Comparison of the no NMDA control versus +NMDA and the +NMDA versus NMDA + MK801/APV in FAE slices revealed the difference was not significant (ns). U0126 pretreated slices were significantly different from untreated control slices in Sacc and FAE groups (# $p<0.001$). Data from +U0126 versus +NMDA + U0126 slices were not significantly different.

Fig. 4.

ERK2 phosphorylation in response to treatment with phorbol 12,13 diacetate (PDA), $n=6$, or forskolin (FSK), $n=8$, in saccharin control (Sacc) and fetal alcohol exposed (FAE) adult hippocampal slices. (A) Representative phospho-ERK1/2 (pERK1/2; top) and total ERK1/2 (bottom) Western blots for both Sacc and FAE hippocampal slices treated without or with 3 μ M PDA. (B) ERK2 data expressed as a ratio of pERK2 to total ERK2 in Sacc and FAE hippocampal slices treated without or with 3 μ M PDA. Two-way ANOVA of the data revealed a significant effect of PDA treatment ($p<0.01$). Post hoc analysis showed a significant ERK2 activation upon PDA treatment in both groups, $**p<0.01$. (C) Representative phospho-ERK1/2 (pERK1/2) top and total ERK1/2 (bottom) Western blots for both Sacc and FAE hippocampal slices treated without or with 50 μ M FSK. (D) ERK2 data expressed as a ratio of pERK2 to total ERK2 in Sacc and FAE hippocampal slices treated with 50 μ M FSK. Two-way ANOVA of the data revealed a significant effect of FSK treatment ($p<0.01$). Post hoc analysis showed a significant ERK2 activation upon FSK treatment in both groups, $**p<0.01$.

Fig. 5. NMDA-induced activation of ERK1/2 in re-sectioned hippocampal slices stimulated *in vitro* (scale bar = 300 μ m). (A) Representative 4X images of Sacc and FAE slices showing anti-phospho-ERK1/2 immunoreactivity (red) in 16 μ m hippocampal slices without NMDA stimulation (-NMDA) or after exposure to 100 μ M NMDA for 3 min (+NMDA). Regions indicated are CA1, dentate gyrus (DG) and CA3. NMDA-mediated ERK1/2 activation was shown to occur mainly in CA1 region for both groups. (B) No significant difference in area of ERK1/2 activation found between Sacc (n=5) and FAE (n=6) slides. DAPI (blue) was used to stain nuclei.

Fig. 6. NMDA induced activation of ERK1/2 in re-sectioned hippocampal slices stimulated *in vitro*. Representative 10X images of CA1, dentate gyrus (DG) and CA3 regions without NMDA (-NMDA) or after exposure to 100 μ M NMDA for 3 min (+NMDA) for Sacc (A) and FAE (B) groups (scale bar = 100 μ m). The majority of ERK1/2 activation was found to occur in CA1 pyramidal dendrites within the stratum radiatum (s.r.) for both Sacc and FAE images. Stratum pyramidale (s.p.) and stratum oriens (s.o.) also shown. ERK1/2 activation in the DG region is depicted by white arrows on Sacc 10X image (A). DAPI (blue) was used to stain nuclei.

Fig. 7. ERK1/2 activation in CA1 (A) and dentate gyrus (DG) (B) for Sacc (n=5) and FAE (n=6) re-sectioned slices (-NMDA and +NMDA). (A) Two-way ANOVA of CA1 data showed a significant effect of NMDA treatment ($p < 0.0001$). Post hoc analysis revealed a significant increase in both Sacc ($***p < 0.0001$) and FAE ($**p < 0.01$) groups upon NMDA stimulation in the CA1 region. (B) Two-way ANOVA of DG data showed a significant effect of NMDA treatment ($p < 0.05$) and a significant effect of prenatal diet ($p < 0.05$), however there was not a significant interaction. Post hoc analysis revealed a significant increase in ERK1/2 activation in the DG of Sacc slices ($*p < 0.05$), but not in FAE slices.