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Impact of Combined Prenatal Ethanol and Prenatal Stress Exposures on Markers of Activity-Dependent Synaptic Plasticity in Rat Dentate Gyrus

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Abstract

Prenatal ethanol exposure and prenatal stress can each cause long-lasting deficits in hippocampal synaptic plasticity and disrupt learning and memory processes. However, the mechanisms underlying these perturbations following a learning event are still poorly understood. We examined the effects of prenatal ethanol exposure and prenatal stress exposure, either alone or in combination, on the cytosolic expression of activity-regulated cytoskeletal (ARC) protein and the synaptosomal expression of AMPA-glutamate receptor subunits (GluA1 and GluA2) in dentate gyrus of female adult offspring under baseline conditions and after 2-trial trace conditioning (TTTC). Surprisingly, baseline cytoplasmic ARC expression was significantly elevated in both prenatal treatment groups. In contrast, synaptosomal GluA1 receptor subunit expression was decreased in both prenatal treatment groups. GluA2 subunit expression was elevated in the prenatal stress group. TTTC did not alter ARC levels compared to an unpaired behavioral control (UPC) group in any of the 4 prenatal treatment groups. In contrast, TTTC significantly elevated both synaptosomal GluA1 and GluA2 subunit expression relative to the UPC group in control offspring, an effect that was not observed in any of the other 3 prenatal treatment groups. Given ARC's role in regulating synaptosomal AMPA receptors, these results suggest that prenatal ethanol-induced or prenatal stress exposure-induced increases in baseline ARC levels could contribute to reductions in both baseline and activity-dependent changes in AMPA receptors in a manner that diminishes the role of AMPA receptors in dentate gyrus synaptic plasticity and hippocampal-sensitive learning.

Keywords

fetal alcohol spectrum disorder; prenatal stress; AMPA receptor; ARC; Western blot

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Introduction

Prenatal exposure to ethanol results in long-lasting deleterious effects on IQ, behavior, attention and, in cases of more severe exposures, physical dysmorphology. The severity of ethanol's impact on the developing fetus is related to the quantity of ethanol consumed, the pattern of ethanol consumption (Bailey et al., 2004), and the gestational timing of exposure (Kelly, Day, & Streissguth, 2000; Sood et al., 2001). The resulting range of outcomes has been termed fetal alcohol spectrum disorder (FASD). In the US, an estimated 2–5% of school-aged children suffer from FASD (May et al., 2009), the majority of which have no physical characteristics, but demonstrate significant reductions in IQ and cognitive deficits (Green et al., 2009; Kodituwakku, 2009; Willoughby, Sheard, Nash, & Rovet, 2008). These reductions in cognition persist into adulthood (Barr et al., 2006), signifying a long-lasting role of ethanol exposure during development on learning and memory.

Various animal models of prenatal ethanol exposure have been employed to elucidate the teratogenic mechanisms of ethanol in the developing fetus (Cudd, 2005). These models allow for precise experimental control, as well as providing for potential cellular and molecular analysis of animals demonstrating behavioral deficits, which parallel those observed in humans. Studies of adult animals exposed to moderate amounts of ethanol during gestation have reported reductions in hippocampus-sensitive learning and memory tasks (Berman & Hannigan, 2000; Brady, Allan, & Caldwell, 2012; Savage, Becher, de la Torre, & Sutherland, 2002; Savage et al., 2010; Staples, Rosenberg, Allen, Porch, & Savage, 2013; Sutherland, McDonald, & Savage, 2000; Weeber, Savage, Sutherland, & Caldwell, 2001), as well as deficits in measures of hippocampal synaptic plasticity, an important process associated with learning (Brady et al., 2013; Savage et al., 2002; Sutherland, McDonald, & Savage, 1997; Titterness & Christie, 2012; Varaschin, Akers, Rosenberg, Hamilton, & Savage, 2010).

Similar to ethanol, maternal stress during pregnancy can also cause adverse neurodevelopment in the fetus. In human populations, children born to women who have survived acute trauma (severe weather events, terrorist attacks, etc.) or have adverse living circumstances (chronic stress) have been reported to suffer from significant cognitive impairments (Laplante et al., 2004; Martini, Knappe, Beesdo-Baum, Lieb, & Wittchen, 2010; O'Connor, Heron, Golding, Glover, & ALSPAC Study Team, 2003; Rodriguez & Bohlin, 2005). However, identifying and characterizing stress in human populations is challenging due to the high number of variables associated with individual perceptions of stress. Thus, most knowledge regarding the effects of prenatal stress on offspring behavior has come from animal model studies. Animals born to mothers who were exposed to stressors during gestation are typically smaller (Rice et al., 2010), demonstrate heightened anxiety and stress reactivity (Vallée et al., 1997), and display cognitive deficits, particularly on hippocampal-sensitive tasks, as well as long-term potentiation (Yang, Han, Cao, Li, & Xu, 2006). The severity of these characteristics is related to the type and duration of the stress the mother was subjected to during her pregnancy (Mychasiuk, Illytskyy, Kovalchuk, Kolb, & Gibb, 2011; O'Connor et al., 2003), as well as her predisposition to stress reactivity.

While a great deal is known regarding the neurochemical changes associated with synaptic strengthening in long-term potentiation (see review by Shepherd & Huganir, 2007), the neurochemical mechanisms underlying the deficits in activity-dependent synaptic plasticity as a consequence of either prenatal ethanol exposure or prenatal stress are not well understood. The quantity, activity, and/or translocation of a number of proteins are critical to the expression or maintenance of long-term potentiation (LTP), thought to be one cellular mechanism underlying learning (see review by Citri & Malenka, 2008). In the present study, we examined the impact of either prenatal ethanol exposure and/or prenatal stress exposure on the expression of 2 proteins involved in activity-dependent changes in synaptic plasticity, namely, activity-regulated cytosolic protein (ARC) and 2 AMPA-glutamate receptor subunits (GluA1 and GluR2). ARC protein is up-regulated within 4 h following maximal electroconvulsive shock (MECS) (Lyford et al., 1995), immediately following classical eye-blink conditioning (Mokin, Lindahl, & Keifer, 2005), 1 h following auditory-cued fear conditioning (Lonergan, Gafford, Jarome, & Helmstetter, 2010), and 1 h following the final training in a lever-press task (Kelly & Deadwyler, 2003). ARC has direct roles in expanding the cytoskeletal network within a postsynaptic cell (Huang, Chotiner, & Steward, 2007). Likewise, previous work has demonstrated activity-dependent increases in AMPA receptors following the induction of LTP (Shi, Hayashi, Esteban, & Malinow, 2001; Takahashi, Svoboda, & Malinow, 2003), with specific increases observed in GluA1 and GluA2 expression following various forms of fear-conditioned learning (Matsuo, Reijmers, & Mayford, 2008; Yeh, Mao, Lin, & Gean, 2006). Interestingly, the interaction of ARC protein expression and the AMPA receptor has implications of various forms of synaptic plasticity. While ARC is known to play a role in expanding the actin cytoskeleton network, it also has been demonstrated to play a role in AMPA receptor endocytosis from the synaptic membrane (Chowdhury et al., 2006), implicating a role for long-term depression (LTD) (Waung, Pfeiffer, Nosyreva, Ronesi, & Huber, 2008).

As described above, either prenatal ethanol or prenatal stress exposure can separately induce deficits in LTP and learning. ARC expression and AMPA receptor expression are required for proficient performance on learning tasks, with recent evidence demonstrating an elevation in ARC expression following various models of trace fear conditioning (Chau, Prakapenka, Fleming, Davis, & Galvez, 2013; Chia & Otto, 2013) as well as the expression of LTP (Plath et al., 2006). In models of prenatal ethanol exposure, basal quantities of AMPA receptor subunits are differentially expressed as compared to non-exposed controls (Dettmer et al., 2003), and ARC mRNA expression is reduced as compared to controls following a novel social experience (Hamilton et al., 2010). However, basal ARC protein expression has not been evaluated in animals prenatally exposed to ethanol, stress, or a combination of the two. Similarly, changes in the expression of ARC protein or AMPA receptor subunits following behavioral activation has not been examined in animals prenatally exposed to ethanol, stress, or the combination of these 2 prenatal exposure paradigms.

Using a novel model of combined prenatal ethanol and prenatal stress exposure, we recently reported that moderate prenatal ethanol exposure decreased learning of a behaviorally challenging TTTC paradigm (Staples et al., 2013). Employing the training portion of TTTC as a behavioral activation paradigm, we tested the hypothesis that prenatal ethanol exposure

and/or prenatal stress exposure would diminish TTTC-induced elevations in ARC- and AMPA-receptor subunit expression.

Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. All animal care and usage complied with the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

Prenatal ethanol and prenatal stress paradigms

Prenatal ethanol-exposed and prenatal stress-exposed rat offspring were generated as previously described by Staples et al. (2013). Briefly, Long-Evans females and breeder males (Harlan Industries, Indianapolis, IN) were single-housed and maintained on a reverse light/dark cycle. Prior to mating, females were gradually acclimated to voluntary drinking of 5% ethanol in 0.066% saccharin (Sacc) for 4 h during the awake phase of the light/dark cycle. Tap water and rat chow were available *ad libitum*. Following 2 weeks of 5% ethanol consumption, females drinking within 1 standard deviation of the group mean were paired with a proven breeder male until pregnancy was confirmed via the presence of a vaginal plug. Female rats did not consume ethanol during the breeding procedure. Pregnant rat dams were then returned to their single-housed condition and voluntarily consumed either 0% or 5% ethanol in 0.066% saccharin until delivery.

Prenatal stress was elicited by mimicking exposure to a predator scent. Accordingly, rat dams assigned to the prenatal stress group were exposed to 3% 2,3,5-trimethyl-3-thiazoline (TMT, Contech, Victoria, BC, Canada) for 20 min at 1600 hours (4 h following the removal of the ethanol or saccharin drinking tube) on gestational days (GD) 13, 15, 17, and 19 (see Fig. 1), because evidence has demonstrated that developing rodent fetuses are only susceptible to stress during the third week of gestation (Diaz, Brown, & Seckl, 1998). This paradigm has been shown to elevate maternal serum corticosterone levels 3-fold over non-stressed controls, and the rat dams do not acclimate to this stressor with repeated TMT exposure episodes (Staples et al., 2013). Additionally, this prenatal stress paradigm does not elicit significant growth deficits in the offspring, as noted in Staples et al. (2013), thereby reducing the number of potential confounding factors. Following delivery, dams and litters were left undisturbed until weaning on postnatal day 25 when the offspring were moved into sex-matched group housing with littermates. Except for routine cage bedding changes, offspring were left undisturbed until experimental use. Only female offspring at 3 months of age were used in the experiments described below due to their improved capacity to acquire trace conditioning learning as compared to age-matched male counterparts (Dalla, Papachristos, Whetstone, & Shors, 2009).

Basal ARC and GluA receptor expression

Six female subjects from each of the 4 prenatal treatment groups were transferred to single-housed caging. In order to reduce estrus cycle variations in estrogen, which can create confounds in analyzing data derived from female subjects, all female offspring were exposed to used male bedding 24 h prior to sacrifice to synchronize their estrous cycles.

Estrous cycle synchronicity was confirmed in a separate set of females exposed to the used male bedding in an identical manner in order to avoid confounds from the stress of vaginal cytological sampling in the behaviorally induced expression of the neuronal markers of interest.

Tissue preparation was similar to the procedures described by Samudio-Ruiz, Allan, Sheema, & Caldwell (2010), as summarized in Fig. 2. Rats were rapidly decapitated and the cortex was quickly removed from the skull. The dentate gyrus (DG) portion of the hippocampus was micro-dissected from the surrounding cortical tissue, frozen in liquid nitrogen, and stored at -80°C until further processing. The 2 DG from a single animal were pooled to serve as a single sample and were frozen, stored, and processed together. Subsequently, tissue samples were thawed on ice, then homogenized in a glass Dounce homogenizer in 300 μL of homogenization buffer (320 mM Sucrose, 10 mM Tris HCl, 1 mM NaVO_4 , 5 mM NaF, 1 mM EDTA, 1 mM EGTA, and protease inhibitor [Roche, Indianapolis, IN]) with 6 up and down strokes each of a “loose-fitting” and then a tight-fitting pestle. The resulting homogenate was centrifuged at $1,000 \times g$ for 6 min at 4°C . The supernatant (S1) was then centrifuged at $10,000 \times g$ for 15 min at 4°C , producing a crude synaptosomal fraction (P2) and a crude cytosolic fraction (S2). These electron micrographic and biochemical characterizations of these fractions have been reported previously (Dunah & Standaert, 2001; Huttner, Schiebler, Greengard, & De Camilli, 1983). One percent sodium dodecyl sulfate (SDS) was added to each sample and the protein concentration was determined by BCA protein assay (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Homogenized samples were frozen in liquid nitrogen and stored at -80°C until Western blot analysis.

A total of 10 μg of each sample was loaded onto 8% SDS polyacrylamide gel and electrophoresed at 150 V for 1 h, then transferred to a PVDF membrane at 100 V for 1 h at room temperature or at 30 V overnight at 4°C . Membranes were then washed in Tris-buffered Saline (TBS) for 5 min and then blocked in iBlock (Life Technologies, Grand Island, NY) for 1 h at room temperature. Subsequently, membranes were incubated in primary antibody (ARC-1:150, Santa Cruz Biotechnology #17839; GluA1-1:600, Millipore #AB1504; or GluR2-1:500, Abcam #AB20673) and 0.1% Tween in iBlock for 1 h at room temperature or overnight at 4°C . Membranes were washed for 3×5 min in TBS-Tween (TBST) and then incubated in the appropriate secondary fluorescent antibody (1:10,000 anti-rabbit or 3:10,000 anti-mouse, LiCor Biosystems, Lincoln, NE) in iBlock with 0.1% Tween and 0.01% SDS for 45 min at room temperature. Finally, the membranes were washed 3×5 min in TBST and 2×5 min in TBS. Membranes were scanned using an infrared imaging system (Li-Cor Odyssey, Li-Cor Biosciences, Lincoln, NE) at the 800 nm wavelength.

Blots were then incubated with 0.4% Coomassie stain (Coomassie Brilliant Blue R-250, Bio-Rad, Hercules, CA) in 50% methanol, 10% acetic acid, and 40% ddH_2O for 30 min at room temperature. The membranes were then destained using 50% ddH_2O , 43% methanol, and 7% acetic acid. Destaining solution was exchanged for fresh solution until protein bands were clearly resolved. The blot was then dried and scanned on a flatbed scanner (Canon, Melville NY). Optical densities of proteins of interest and Coomassie staining were measured using Image Studio Lite (Li-Cor Biosciences, Lincoln, NE). The optical density of

each band was first normalized to the Coomassie staining of the lane to control for variation in total protein loaded (Welinder & Ekblad, 2011), then normalized to a hippocampal homogenate loading control which was present on all blots to control for differences in incubations between blots.

ARC and GluA receptor expression after 2-trial trace conditioning

Twelve subjects from each of the 4 prenatal treatment groups were randomly assigned into 2 behavioral condition groups (6 rats per prenatal treatment/behavioral condition group). Subjects were maintained in single-housed cages for 1 week and exposed to used male bedding 24 h prior to behavioral testing.

One behavioral group of subjects was exposed to the training portion of a 2-Trial Trace Conditioning (TTTC) paradigm as described by Staples et al. (2013). This associative learning task was preferred as a “behavioral activation” procedure, given the relatively short period of time during which the learning occurs as well as its greater sensitivity to functional damage in the hippocampal formation, compared to standard 1-trial contextual delayed fear conditioning. Briefly, each rat was placed in the conditioning chamber, which consisted of a Habitest System (Coulbourn Instruments, Allentown, PA) equipped with a Precision Regulated Shocker and a 12" W × 10" D × 12" H test cage having a metal grid floor. Subjects were allowed to acclimate to the novel surroundings for 3 min. Immediately following the acclimation period, a constant, 30-sec, 90-dB tone was played. After a 60-sec trace interval where no stimulus was present, a 2-sec, 1-mA foot shock was administered. Ninety seconds later, the tone-trace-foot shock pairing was repeated. One minute following the second foot shock, the rat was moved back to her home cage and transferred back to her home room.

Female offspring in the second behavioral group served as a behavioral control for the TTTC procedure. These rats were housed for 1 week prior to experimentation in plastic cages that had been modified with metal grid rods across the cage floor, approximately 0.5 cm above the bottom of the cage. On the testing day, these rats were subjected to all 3 of the relevant stimuli (context, tone, and foot shock), but at temporally and contextually dissociated points in time to prevent the formation of association among the 3 stimuli. These so-called “Unpaired Control” (UPC) rats were allowed to explore the context chamber for 8.5 min and then were returned to their grid floor home cages. One hour later, the UPC animals were given 2 presentations of the tone while remaining in their grid floor cages. Subsequently, the rats were moved back to the home housing room. One hour later, each UPC rat was shocked twice in her grid floor cage, while in her home room environment.

For both behavioral condition groups, rats were sacrificed 1 h following the second foot shock. This time point was selected based on a preliminary time-course study using untreated control rats, which established that 60 min post-TTTC training was an optimal time to measure both ARC- and AMPA-receptor subunit expression (unpublished observations). Following sacrifice, tissues were processed and Western blot analyses were performed as described above.

Statistical analysis

Statistical analysis was conducted using SigmaPlot 11.0 (San Jose, CA). Graphs were generated using GraphPad 6.0 (La Jolla, CA).

Results

Impact of the dual prenatal exposure paradigm on maternal status and offspring

Pregnant dams consumed an average of 1.89 ± 0.09 g ethanol/kg body weight/gestational day. Voluntary consumption of 5% ethanol has been reported to produce a peak maternal serum ethanol concentration of 60.8 ± 5.8 mg/dL. There was no significant reduction in maternal ethanol consumption as a result of stress exposure or maternal stress response as a result of ethanol consumption, the quantity of weight gained by dams during gestation, offspring birth weight, measures of maternal care, litter size, or pup growth to weaning (Staples et al., 2013). Prenatal ethanol-exposed offspring generated from this paradigm demonstrated a significant (33%) reduction in behavioral freezing to the context on the TTTC task whereas prenatal stress exposure alone did not significantly impair learning (Staples et al., 2013).

Baseline ARC and GluA receptor subunit levels

Cytosolic ARC expression

A 2-way ANOVA analysis revealed an interactive effect of prenatal ethanol and prenatal stress exposures ($F[1,19] = 9.83, p = 0.005$) on baseline cytoplasmic ARC levels (Fig. 3). *Post hoc* comparisons revealed a significant elevation in basal ARC expression in rats exposed to either prenatal stress or prenatal ethanol as compared to the Sacc/No Stress control group ($p = 0.013$ and $p = 0.003$, respectively) and a nearly significant elevation due to combined prenatal exposures as compared to the Sacc/No Stress controls ($p = 0.07$).

Synaptosomal GluA1 expression

A 2-way ANOVA analysis revealed a main effect of prenatal ethanol exposure ($F[1,19] = 7.68, p = 0.01$), and a trend toward a significant main effect of prenatal stress ($F[1,19] = 3.37, p = 0.08$) (Fig. 4A). *Post hoc* comparisons revealed a significant reduction in baseline GluA1 expression in the synaptosomal fraction of rats exposed to dual prenatal ethanol and prenatal stress exposure as compared to animals not exposed to ethanol or stress ($p = 0.005$) or only prenatal stress ($p = 0.038$).

Synaptosomal GluA2 expression

A 2-way ANOVA analysis revealed a main effect of prenatal stress exposure ($F[1,20] = 8.31, p = 0.009$) (Fig. 4B). *Post hoc* comparisons revealed a significant elevation in basal GluA2 expression in the synaptosomal fraction in animals exposed to only prenatal stress as compared to Sacc/No Stress animals ($p = 0.035$).

The sum of GluA1 and GluA2 expression in the synaptosomal fraction for the 4 exposure groups was determined by adjusting the optical density of each group normalized to the corresponding Sacc/No Stress control group optical density. A 3-way ANOVA analysis

(ethanol $\times$ stress $\times$ subunit) revealed significant main effects of prenatal ethanol exposure ($F[1,40] = 10.5, p = 0.002$) and subunit ($F[1,40] = 13.0, p < 0.001$), and a significant interactive effect of prenatal stress exposure and subunit ($F[1,40] = 12.6, p = 0.001$) (Fig. 4C). *Post hoc* comparisons revealed a significant reduction in the combined total quantity of GluA1 and GluA2 in the synaptosomal fraction of the Ethanol/No Stress and Ethanol/Stress exposure groups as compared to the Sacc/No Stress group ($p < 0.05$ for both comparisons), as well as a significant reduction in the quantity of GluA1 in the synaptosomal fraction in Ethanol/Stress when compared to the Sacc/No Stress group ($p < 0.05$).

Impact of TTTC on ARC and GluA receptor subunit expression

Cytosolic ARC

Expression after TTTC: ARC levels in each of the 4 prenatal treatment groups were similar in the UPC control rats (Fig. 5) in comparison to the naïve unhandled baseline control rats reported in Fig. 3. A 3-way ANOVA analysis (ethanol $\times$ stress $\times$ training) revealed a main effect of prenatal ethanol ($F[1,37] = 7.56, p = 0.009$), and a main interaction of ethanol and stress ($F[1,37] = 47.2, p < 0.001$) (Fig. 5). *Post hoc* comparisons revealed significant elevations in ARC protein because of each prenatal exposure condition as compared to its Sacc/No Stress UPC behavioral control ($p < 0.05$ for all measures). However, there were no significant alterations in ARC protein expression because of TTTC compared to the UPC control group for any prenatal exposure group, including the Sacc/No Stress group (Fig. 5).

Synaptosomal GluA1 expression after TTTC: A 3-way ANOVA analysis (ethanol $\times$ stress $\times$ training) revealed a main effect of prenatal ethanol exposure ($F[1,40] = 9.52, p = 0.004$), a main interaction of ethanol and stress ($F[1,40] = 10.8, p = 0.002$), and a nearly significant main effect of training ($F[1,40] = 3.07, p = 0.08$) (Fig. 6A). *Post hoc* comparisons revealed a significant elevation because of TTTC in GluA1 expression in the Sacc/No Stress animals ($p = 0.04$), an effect that was not observed in any other prenatal exposure group following TTTC.

Synaptosomal GluA2 expression after TTTC: A 3-way ANOVA analysis (ethanol $\times$ stress $\times$ training) revealed a main effect of prenatal stress exposure ($F[1,38] = 5.17, p = 0.029$) and a main interaction effect of ethanol $\times$ stress $\times$ training ($F[1,38] = 4.76, p = 0.035$) (Fig. 6B). *Post hoc* comparisons revealed a significant elevation in GluA2 expression in the Sacc/No Stress animals ($p = 0.05$) that was not observed in any other prenatal exposure group.

Discussion

There are 4 salient observations based on the work presented in this report. As hypothesized, prenatal ethanol exposure and/or prenatal stress exposure abolished activity-dependent increases in synaptosomal GluA1 and GluA2 in the dentate gyrus. However, in contrast to our predictions, two-trial trace conditioning did not increase ARC levels and prenatal ethanol and/or prenatal stress significantly increased basal levels of ARC and interactively reduced basal synaptosomal GluA1 levels in patterns not consistently observed for GluA2.

At the onset of this project, we had predicted that the basal measures of ARC and GluA subunits would not be affected by either prenatal ethanol or prenatal stress exposure, and any observable differences would more likely occur as a function of activity-dependent conditions. These predictions were based on prior observations that moderate prenatal ethanol exposure did not affect the density of specific [^3H]-fluorowillardiine binding to AMPA receptors (Savage et al., 1992), and that dentate gyrus baseline input/output curves, as a measure of AMPA receptor responsiveness, are not altered in prenatal ethanol-exposed offspring (Sutherland et al., 1997; Varaschin et al., 2010).

In contrast to our prediction, both prenatal ethanol and prenatal stress exposure resulted in 3-fold increases in basal cytosolic ARC protein levels (Fig. 3). No further increase in basal ARC expression was observed in the dual exposure group. Currently, there appears to be no evidence in the literature to suggest a mechanistic basis for increased basal ARC expression in these prenatal treatment groups. However, given that both prenatal ethanol (Kelly, Mahoney, Randich, & West, 1991; Lan et al., 2006; Weinberg, 1988) and prenatal stress (Maccari et al., 2003; Vallée et al., 1997) have been shown to elevate HPA axis responsiveness to stressors, and that single housing and social isolation have been shown to increase stress hormones (Stranahan, Khalil, & Gould, 2006; Weiss, Pryce, Jongen-Rêlo, Nanz-Bahr, & Feldon, 2004), it is possible that maintenance in the single-housed cage environment during the last week prior to sacrifice caused sufficient stress to increase neural activity in a manner that differentially elevated ARC levels in otherwise naïve unhandled control conditions. Future work should include an assessment of the impact of single-housing stress on learning in the TTTC task, as well as a comparison of the expression of ARC protein and AMPA receptor subunit expression in animals group-housed compared to isolated animals. Similarly, future work should also include electrophysiological investigations on the capacity of these animals for LTD, as ARC protein is critical for LTD, and has been shown to be involved in the removal of AMPA receptors from the membrane during LTD stimulation. Either scenario, or potentially both, could provide additional insights into the interpretation of the present data.

The second unexpected observation from this study was that both prenatal ethanol exposure and prenatal stress reduced baseline levels of synaptosomal GluA1 receptor subunit expression (Fig. 4A). In this case, the dual exposure paradigm produced an interactive effect. Curiously, prenatal stress exposure alone elevated GluA2 level, an effect that was not observed with prenatal ethanol exposure alone or in the dual exposure paradigm (Fig. 4B). However, prenatal ethanol exposure significantly reduced the sum total of synaptosomal GluA1 and GluA2 (Fig. 4C), whereas the sum was not different in the prenatal stress group. Our results in prenatal ethanol-exposed offspring are supported by Bellinger, Davidson, Bedi, & Wilce (2002), where early postnatal ethanol exposure resulting in peak serum ethanol concentrations of 330 mg/dL reduced both GluA1 and GluA2 subunit levels in cortex. The differences in the GluA2 outcomes between our study and prior reports may be attributable to the timing of ethanol exposure during development, the difference in peak serum ethanol concentrations, and/or the brain region and age of analysis in the 2 studies.

A third unexpected outcome in these studies was the lack of an effect of TTTC on ARC expression levels in the dentate gyrus (Fig. 5). This outcome is in contrast to previous

studies by others demonstrating significant increases in ARC levels following various behavioral challenges. For example, Kelly & Deadwyler (2003) observed significant elevations in cerebral cortical ARC levels following multiple days of training in a lever press. Mokin et al. (2005) reported significant elevations in ARC protein in turtle brain stem following classical eye-blink conditioning. Lonergan et al. (2010) found significant elevations in ARC protein in dorsal hippocampus of males sampled 1 h following 4 trials of auditory-cued fear conditioning. Thus, differences in the nature, duration, or intensity of a behavioral challenge, the brain regions sampled, and other factors such as gender may produce differential ARC responses. Relative to our results, it is possible that the TTTC paradigm employed in the present study was an insufficient behavioral challenge to induce an activity-dependent elevation in ARC in the dentate gyrus.

In contrast to the effects of TTTC on ARC expression levels, both synaptosomal GluA1 (Fig. 6A) and GluA2 (Fig. 6B) were significantly elevated in Sacc/No Stress controls after TTTC in comparison to their respective UPC control groups. This effect is consistent with prior studies using electrophysiological (Shi et al., 1999) and behavioral stimulation (Schiapparelli, Simón, Del Río, & Frechilla, 2006; Shi et al. 1999). Shi et al. (1999) tetanically stimulated hippocampal primary neurons in culture and observed that GFP-tagged GluA1 was delivered to the dendritic spine within 30 min of stimulation, while Schiapparelli et al. (2006) measured an elevation in GluA1 in the membrane fraction of the hippocampus in animals 1 h after training in a passive avoidance task. As predicted, and in contrast to the Sacc/No Stress control prenatal treatment group, TTTC did not increase either GluA1 or GluA2 levels in any of the other 3 prenatal treatment conditions. These results contrast to a prior report where using restraint stress 3 times a day for 45 min in each session during the last week of gestation did not affect activity-dependent changes in GluA1 or GluA2 receptor subunit expression in the prefrontal cortices of rats (Fumagalli et al., 2009).

Taken together, these findings imply that exposure to prenatal ethanol and prenatal stress alters the biochemical machinery underlying learning in a hippocampal-sensitive task. At the present time, there is a paucity of information in the literature to help interpret how the results of these experiments shed light on putative underlying mechanisms contributing to the synaptic plasticity deficits in dentate gyrus of prenatal ethanol-exposed or prenatal stress-exposed offspring. Given that ARC has been associated with the endocytosis of AMPA receptors from the cellular membrane (Chowdhury et al., 2006), one parsimonious explanation that incorporates the salient observations in this study is the notion that the 3-fold increase in basal cytoplasmic ARC levels (Fig. 3) disrupted the cycling of AMPA receptor subunits in a manner that reduced basal levels of synaptosomal GluA1 (Fig. 4A) and interfered with behavioral activation-dependent increases in both synaptosomal GluA1 and GluA2 in the dentate gyrus. As GluA1 is required for the initiation of LTP (Sanderson et al., 2008), this putative mechanism provides one explanation for the deficits in dentate gyrus LTP observed in prenatal ethanol-exposed (Brady et al., 2013; Sutherland et al., 1997; Titterness & Christie, 2012; Varaschin et al., 2010) and prenatal stress-exposed (Titterness & Christie, 2012) offspring. Further, reductions in GluA subunit expression have been also associated with reduced performance on object recognition and passive avoidance tests (Schiapparelli et al., 2006), and animals lacking the GluA1 subunit have reduced

performance on hippocampal-sensitive tasks such as radial arm maze and a non-matching to place T-maze, 2 measures of working memory (Sanderson et al., 2008).

Clearly, additional work will be required to better elucidate the mechanistic basis for prenatal ethanol-induced and prenatal stress-induced deficits in synaptic plasticity and learning. For example, one important factor in AMPA receptor-mediated synaptic plasticity is the phosphorylation status of the AMPA receptor protein. Phosphorylation of S845 of the GluA1 subunit results in increased conductivity and inward rectification of ionic current (Banke et al., 2000), while phosphorylation of the S831 site results in increased membrane localization (Hayashi et al., 2000). Thus, future work assessing synaptic plasticity in these models could measure changes in the levels of phosphorylation at these 2 sites under activity-dependent conditions. One limitation of the present work is that only the presence of AMPA receptor subunits was measured, while the functionality of such receptors would be of critical importance. In addition, it is not possible to assess whether the reduction in AMPA receptor subunits reported here is due to internalization of the receptors or restricted delivery of the receptors to the membrane by using Western blotting techniques. Alternative experiments would be required to further elucidate the exact localization and function of the receptors described here.

Beyond AMPA receptor function, baseline and activity-dependent assessment of other glutamate receptor proteins, such as NMDA and mGluR receptors, along with the multiple cellular signaling systems triggered by these receptor subtypes (see review by Medina, 2011), is warranted to gain a clearer insight on how these prenatal insults cause long-lasting changes in the ability of neuronal populations to respond to their environment. For example, there is substantial evidence to show that both prenatal ethanol and prenatal stress can alter the expression, subunit composition, and synaptic membrane localization of NMDA receptors (Barros et al., 2004; Brady et al., 2013; Kinnunen, Koenig, & Bilbe, 2003; Savage, Montano, Otero, & Paxton, 1991). Further, prenatal ethanol exposure has been shown to alter the levels of several phospholipases (Allan, Weeber, Savage, & Caldwell, 1997; Weeber et al., 2001), protein kinase C activity, (Galindo et al., 2004; Perrone-Bizzozero et al., 1998; Tanner et al., 2004) and ERK kinase (Samudio-Ruiz et al., 2010). More comprehensive knowledge of how prenatal insults alter these systems, particularly under activity-dependent conditions, is critical for a clearer understanding of the neurobiological bases of synaptic plasticity deficits, which could subsequently lead to the establishment of more rational therapeutic approaches for ameliorating the synaptic plasticity and learning deficits associated with these neurodevelopmental insults.

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References

- Allan AM, Weeber EJ, Savage DD, Caldwell KK. Effect of prenatal ethanol exposure on phospholipase C-beta 1 and phospholipase A2 in hippocampus and medial frontal cortex of adult rat offspring. *Alcoholism: Clinical and Experimental Research*. 1997; 21:1534–1541.
- Bailey BN, Delaney-Black V, Covington CY, Ager J, Janisse J, Hannigan JH, et al. Prenatal exposure to binge drinking and cognitive and behavioral outcomes at age 7 years. *American Journal of Obstetrics and Gynecology*. 2004; 191:1037–1043. [PubMed: 15467586]
- Banke TG, Bowie D, Lee H, Huganir RL, Schousboe A, Traynelis SF. Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. *The Journal of Neuroscience*. 2000; 20:89–102. [PubMed: 10627585]
- Barr HM, Bookstein FL, O'Malley KD, Connor PD, Huggins JE, Streissguth AP. Binge drinking during pregnancy as a predictor of psychiatric disorders on the structured clinical interview for DSM-IV in young adult offspring. *The American Journal of Psychiatry*. 2006; 163:1061–1065. [PubMed: 16741207]
- Barros VG, Berger MA, Martijena ID, Sarchi MI, Pérez AA, Molina VA, et al. Early adoption modifies the effects of prenatal stress on dopamine and glutamate receptors in adult rat brain. *Journal of Neuroscience Research*. 2004; 76:488–496. [PubMed: 15114621]
- Bellinger FP, Davidson MS, Bedi KS, Wilce PA. Neonatal ethanol exposure reduces AMPA but not NMDA receptor levels in the rat neocortex. *Brain Research Developmental Brain Research*. 2002; 136:77–84. [PubMed: 12036520]
- Berman RF, Hannigan JH. Effects of prenatal alcohol exposure on the hippocampus: spatial behavior, electrophysiology, and neuroanatomy. *Hippocampus*. 2000; 10:94–110. [PubMed: 10706221]
- Brady ML, Allan AM, Caldwell KK. A limited access mouse model of prenatal alcohol exposure that produces long-lasting deficits in hippocampal-dependent learning and memory. *Alcoholism: Clinical and Experimental Research*. 2012; 36:457–466.
- Brady ML, Diaz MR, Iuso A, Everett JC, Valenzuela CF, Caldwell KK. Moderate prenatal alcohol exposure reduces plasticity and alters NMDA receptor subunit composition in the dentate gyrus. *The Journal of Neuroscience*. 2013; 33:1062–1067. [PubMed: 23325244]
- Chau LS, Prakapenka A, Fleming SA, Davis AS, Galvez R. Elevated Arc/Arg 3.1 protein expression in the basolateral amygdala following auditory trace-cued fear conditioning. *Neurobiology of Learning and Memory*. 2013; 106:127–133. [PubMed: 23891993]
- Chia C, Otto T. Hippocampal Arc (Arg3.1) expression is induced by memory recall and required for memory reconsolidation in trace fear conditioning. *Neurobiology of Learning and Memory*. 2013; 106:48–55. [PubMed: 23872190]
- Chowdhury S, Shepherd JD, Okuno H, Lyford G, Petralia RS, Plath N, et al. Arc/Arg3.1 interacts with the endocytic machinery to regulate AMPA receptor trafficking. *Neuron*. 2006; 52:445–459. [PubMed: 17088211]
- Citri A, Malenka RC. Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology*. 2008; 33:18–41. [PubMed: 17728696]
- Cudd TA. Animal model systems for the study of alcohol teratology. *Experimental Biology and Medicine*. 2005; 230:389–393. [PubMed: 15956768]
- Dalla C, Papachristos EB, Whetstone AS, Shors TJ. Female rats learn trace memories better than male rats and consequently retain a greater proportion of new neurons in their hippocampi. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:2927–2932. [PubMed: 19188598]
- Dettmer TS, Barnes A, Iqbal U, Bailey CD, Reynolds JN, Brien JF, et al. Chronic prenatal ethanol exposure alters ionotropic glutamate receptor subunit protein levels in the adult guinea pig cerebral cortex. *Alcoholism: Clinical and Experimental Research*. 2003; 27:677–681.
- Diaz R, Brown RW, Seckl JR. Distinct ontogeny of glucocorticoid and mineralocorticoid receptor and 11beta-hydroxysteroid dehydrogenase types I and II mRNAs in the fetal rat brain suggest a complex control of glucocorticoid actions. *The Journal of Neuroscience*. 1998; 18:2570–2580. [PubMed: 9502816]

- Dunah AW, Standaert DG. Dopamine D1 receptor-dependent trafficking of striatal NMDA glutamate receptors to the postsynaptic membrane. *The Journal of Neuroscience*. 2001; 21:5546–5558. [PubMed: 11466426]
- Fumagalli F, Pasini M, Frasca A, Drago F, Racagni G, Riva MA. Prenatal stress alters glutamatergic system responsiveness in adult rat prefrontal cortex. *Journal of Neurochemistry*. 2009; 109:1733–1744. [PubMed: 19383086]
- Galindo R, Frausto S, Wolff C, Caldwell KK, Perrone-Bizzozero NI, Savage DD. Prenatal ethanol exposure reduces mGluR5 receptor number and function in the dentate gyrus of adult offspring. *Alcoholism: Clinical and Experimental Research*. 2004; 28:1587–1597.
- Green CR, Mihic AM, Nikkel SM, Stade BC, Rasmussen C, Munoz DP, et al. Executive function deficits in children with fetal alcohol spectrum disorders (FASD) measured using the Cambridge Neuropsychological Tests Automated Battery (CANTAB). *Journal of Child Psychology and Psychiatry, and Allied Disciplines*. 2009; 50:688–697.
- Hamilton DA, Candelaria-Cook FT, Akers KG, Rice JP, Maes LI, Rosenberg M, et al. Patterns of social-experience-related c-fos and Arc expression in the frontal cortices of rats exposed to saccharin or moderate levels of ethanol during prenatal brain development. *Behavioural Brain Research*. 2010; 214:66–74. [PubMed: 20570698]
- Hayashi Y, Shi SH, Esteban JA, Piccini A, Poncer JC, Malinow R. Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science*. 2000; 287:2262–2267. [PubMed: 10731148]
- Huang F, Chotiner JK, Steward O. Actin polymerization and ERK phosphorylation are required for Arc/Arg3.1 mRNA targeting to activated synaptic sites on dendrites. *The Journal of Neuroscience*. 2007; 27:9054–9067. [PubMed: 17715342]
- Huttner WB, Schiebler W, Greengard P, De Camilli P. Synapsin I (protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. *The Journal of Cell Biology*. 1983; 96:1374–1388. [PubMed: 6404912]
- Kelly MP, Deadwyler SA. Experience-dependent regulation of the immediate-early gene arc differs across brain regions. *The Journal of Neuroscience*. 2003; 23:6443–6451. [PubMed: 12878684]
- Kelly SJ, Day N, Streissguth AP. Effects of prenatal alcohol exposure on social behavior in humans and other species. *Neurotoxicology and Teratology*. 2000; 22:143–149. [PubMed: 10758343]
- Kelly SJ, Mahoney JC, Randich A, West JR. Indices of stress in rats: effects of sex, perinatal alcohol and artificial rearing. *Physiology & Behavior*. 1991; 49:751–756. [PubMed: 1881980]
- Kinnunen AK, Koenig JJ, Bilbe G. Repeated variable prenatal stress alters pre- and postsynaptic gene expression in the rat frontal pole. *Journal of Neurochemistry*. 2003; 86:736–748. [PubMed: 12859686]
- Kodituwakku PW. Neurocognitive profile in children with fetal alcohol spectrum disorders. *Developmental Disabilities Research Reviews*. 2009; 15:218–224. [PubMed: 19731385]
- Laplanche DP, Barr RG, Brunet A, Galbaud du Fort G, Meaney ML, Saucier JF, et al. Stress during pregnancy affects general intellectual and language functioning in human toddlers. *Pediatric Research*. 2004; 56:400–410. [PubMed: 15240860]
- Lan N, Yamashita F, Halpert AG, Ellis L, Yu WK, Viau V, et al. Prenatal ethanol exposure alters the effects of gonadectomy on hypothalamic-pituitary-adrenal activity in male rats. *Journal of Neuroendocrinology*. 2006; 18:672–684. [PubMed: 16879166]
- Lonergan ME, Gafford GM, Jarome TJ, Helmstetter FJ. Time-dependent expression of Arc and zif268 after acquisition of fear conditioning. *Neural Plasticity*. 2010; 2010:139891. [PubMed: 20592749]
- Lyford GL, Yamagata K, Kaufmann WE, Barnes CA, Sanders LK, Copeland NG, et al. Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron*. 1995; 14:433–445. [PubMed: 7857651]
- Maccari S, Darnaudery M, Morley-Fletcher S, Zúena AR, Cinque C, Van Reeth O. Prenatal stress and long-term consequences: implications of glucocorticoid hormones. *Neuroscience and Biobehavioral Reviews*. 2003; 27:119–127. [PubMed: 12732228]
- Martini J, Knappe S, Beesdo-Baum K, Lieb R, Wittchen HU. Anxiety disorders before birth and self-perceived distress during pregnancy: associations with maternal depression and obstetric, neonatal

and early childhood outcomes. *Early Human Development*. 2010; 86:305–310. [PubMed: 20547016]

- Matsuo N, Reijmers L, Mayford M. Spine-type-specific recruitment of newly synthesized AMPA receptors with learning. *Science*. 2008; 319:1104–1107. [PubMed: 18292343]
- May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley D, Manning M, et al. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Developmental Disabilities Research Reviews*. 2009; 15:176–192. [PubMed: 19731384]
- Medina AE. Fetal alcohol spectrum disorders and abnormal neuronal plasticity. *Neuroscientist*. 2011; 17:274–287. [PubMed: 21383101]
- Mokin M, Lindahl JS, Keifer J. Immediate-early gene-encoded protein Arc is associated with synaptic delivery of GluR4-containing AMPA receptors during in vitro classical conditioning. *Journal of Neurophysiology*. 2005; 95:215–224. [PubMed: 16339507]
- Mychasiuk R, Ilnytsky S, Kovalchuk O, Kolb B, Gibb R. Intensity matters: brain, behaviour and the epigenome of prenatally stressed rats. *Neuroscience*. 2011; 180:105–110. [PubMed: 21335068]
- O'Connor TG, Heron J, Golding J, Glover V. ALSPAC Study Team. Maternal antenatal anxiety and behavioural/emotional problems in children: a test of a programming hypothesis. *Journal of Child Psychology and Psychiatry*. 2003; 44:1025–1036. [PubMed: 14531585]
- Perrone-Bizzozero NI, Isaacson TV, Keidan GM, Eriquat C, Meiri KF, Savage DD, et al. Prenatal ethanol exposure decreases GAP-43 phosphorylation and protein kinase C activity in the hippocampus of adult rat offspring. *Journal of Neurochemistry*. 1998; 71:2104–2111. [PubMed: 9798936]
- Plath N, Ohana O, Dammermann B, Errington ML, Schmitz D, Gross C, et al. Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. *Neuron*. 2006; 52:437–444. [PubMed: 17088210]
- Rice F, Harold GT, Boivin J, van den Bree M, Hay DF, Thapar A. The links between prenatal stress and offspring development and psychopathology: disentangling environmental and inherited influences. *Psychological Medicine*. 2010; 40:335–345. [PubMed: 19476689]
- Rodriguez A, Bohlin G. Are maternal smoking and stress during pregnancy related to ADHD symptoms in children? *Journal of Child Psychology and Psychiatry, and Allied Disciplines*. 2005; 46:246–254.
- Samudio-Ruiz SL, Allan AM, Sheema S, Caldwell KK. Hippocampal N-methyl-D-aspartate receptor subunit expression profiles in a mouse model of prenatal alcohol exposure. *Alcoholism: Clinical and Experimental Research*. 2010; 34:342–353.
- Sanderson DJ, Good MA, Seeburg PH, Sprengel R, Rawlins JN, Bannerman DM. The role of the GluR-A (GluR1) AMPA receptor subunit in learning and memory. *Progress in Brain Research*. 2008; 169:159–178. [PubMed: 18394473]
- Savage DD, Becher M, de la Torre AJ, Sutherland RJ. Dose-dependent effects of prenatal ethanol exposure on synaptic plasticity and learning in mature offspring. *Alcoholism: Clinical and Experimental Research*. 2002; 26:1752–1758.
- Savage DD, Montano CY, Otero MA, Paxton LL. Prenatal ethanol exposure decreases hippocampal NMDA-sensitive [3H]-glutamate binding site density in 45-day-old rats. *Alcohol*. 1991; 8:193–201. [PubMed: 1648928]
- Savage DD, Rosenberg MJ, Wolff CR, Akers KG, El-Emawy A, Staples MC, et al. Effects of a novel cognition-enhancing agent on fetal ethanol-induced learning deficits. *Alcoholism: Clinical and Experimental Research*. 2010; 34:1793–1802.
- Savage DD, Queen SA, Sanchez CF, Paxton LL, Mahoney JC, Goodlett CR, et al. Prenatal ethanol exposure during the last third of gestation in rat reduces hippocampal NMDA agonist binding site density in 45-day-old offspring. *Alcohol*. 1992; 9:37–41. [PubMed: 1346364]
- Schiapparelli L, Simón AM, Del Río J, Frechilla D. Opposing effects of AMPA and 5-HT1A receptor blockade on passive avoidance and object recognition performance: correlation with AMPA receptor subunit expression in rat hippocampus. *Neuropharmacology*. 2006; 50:897–907. [PubMed: 16620883]

- Shepherd JD, Huganir RL. The cell biology of synaptic plasticity: AMPA receptor trafficking. *Annual Review of Cell and Developmental Biology*. 2007; 23:613–643.
- Shi S, Hayashi Y, Esteban JA, Malinow R. Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell*. 2001; 105:331–343. [PubMed: 11348590]
- Shi SH, Hayashi Y, Petralia RS, Zaman SH, Wenthold RJ, Svoboda K, et al. Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science*. 1999; 284:1811–1816. [PubMed: 10364548]
- Sood B, Delaney-Black V, Covington C, Nordstrom-Klee B, Ager J, Templin T, et al. Prenatal alcohol exposure and childhood behavior at age 6 to 7 years: I. dose-response effect. *Pediatrics*. 2001; 108:E34. [PubMed: 11483844]
- Staples MC, Rosenberg MJ, Allen NA, Porch MW, Savage DD. Impact of combined prenatal ethanol and prenatal stress exposure on anxiety and hippocampal-sensitive learning in adult offspring. *Alcoholism: Clinical and Experimental Research*. 2013; 37:2039–2047.
- Stranahan AM, Khalil D, Gould E. Social isolation delays the positive effects of running on adult neurogenesis. *Nature Neuroscience*. 2006; 9:526–533.
- Sutherland RJ, McDonald RJ, Savage DD. Prenatal exposure to moderate levels of ethanol can have long-lasting effects on hippocampal synaptic plasticity in adult offspring. *Hippocampus*. 1997; 7:232–238. [PubMed: 9136052]
- Sutherland RJ, McDonald RJ, Savage DD. Prenatal exposure to moderate levels of ethanol can have long-lasting effects on learning and memory in adult offspring. *Psychobiology*. 2000; 28:532–539.
- Takahashi T, Svoboda K, Malinow R. Experience strengthening transmission by driving AMPA receptors into synapses. *Science*. 2003; 299:1585–1588. [PubMed: 12624270]
- Tanner DC, Githinji AW, Young EA, Meiri K, Savage DD, Perrone-Bizzozero NI. Fetal alcohol exposure alters GAP-43 phosphorylation and protein kinase C responses to contextual fear conditioning in the hippocampus of adult rat offspring. *Alcoholism: Clinical and Experimental Research*. 2004; 28:113–122.
- Titterness AK, Christie BR. Prenatal ethanol exposure enhances NMDAR-dependent long-term potentiation in the adolescent female dentate gyrus. *Hippocampus*. 2012; 22:69–81. [PubMed: 21080406]
- Vallée M, Mayo W, Dellu F, Le Moal M, Simon H, Maccari S. Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: correlation with stress-induced corticosterone secretion. *The Journal of Neuroscience*. 1997; 17:2626–2636. [PubMed: 9065522]
- Varaschin RK, Akers KG, Rosenberg MJ, Hamilton DA, Savage DD. Effects of the cognition-enhancing agent ABT-239 on fetal ethanol-induced deficits in dentate gyrus synaptic plasticity. *The Journal of Pharmacology and Experimental Therapeutics*. 2010; 334:191–198. [PubMed: 20308329]
- Wang MW, Pfeiffer BE, Nosyreva ED, Ronesi JA, Huber KM. Rapid translation of Arc/Arg3.1 selectively mediates mGluR-dependent LTD through persistent increases in AMPAR endocytosis rate. *Neuron*. 2008; 59:84–97. [PubMed: 18614031]
- Weeber EJ, Savage DD, Sutherland RJ, Caldwell KK. Fear conditioning-induced alterations of phospholipase C-beta1a protein level and enzyme activity in rat hippocampal formation and medial frontal cortex. *Neurobiology of Learning and Memory*. 2001; 76:151–182. [PubMed: 11502147]
- Weinberg J. Hyperresponsiveness to stress: differential effects of prenatal ethanol on males and females. *Alcoholism: Clinical and Experimental Research*. 1988; 12:647–652.
- Weiss IC, Pryce CR, Jongen-Rêlo AL, Nanz-Bahr NI, Feldon J. Effect of social isolation on stress-related behavioural and neuroendocrine state in the rat. *Behavioural Brain Research*. 2004; 152:279–295. [PubMed: 15196796]
- Welinder C, Ekblad L. Coomassie staining as loading control in Western blot analysis. *Journal of Proteome Research*. 2011; 10:1416–1419. [PubMed: 21186791]
- Willoughby KA, Sheard ED, Nash K, Rovet J. Effects of prenatal alcohol exposure on hippocampal volume, verbal learning, and verbal and spatial recall in late childhood. *Journal of the International Neuropsychological Society*. 2008; 14:1022–1033. [PubMed: 18954482]

- Yang J, Han H, Cao J, Li L, Xu L. Prenatal stress modifies hippocampal synaptic plasticity and spatial learning in young rat offspring. *Hippocampus*. 2006; 16:431–436. [PubMed: 16598704]
- Yeh SH, Mao SC, Lin HC, Gean PW. Synaptic expression of glutamate receptor after encoding of fear memory in the rat amygdala. *Molecular Pharmacology*. 2006; 69:299–308. [PubMed: 16219906]

Figure 1.

Schematic diagram illustrating the combined prenatal ethanol and prenatal stress exposure paradigms. Pregnant dams were exposed to either 0% or 5% ethanol in 0.066% saccharin throughout pregnancy. One-half of each group was exposed to 3% predator scent (TMT) on gestational days (GD) 13, 15, 17, and 19. Litters were weaned into sex- and exposure-matched cages on postnatal day (PN) 25. 2-trial trace conditioning and tissue collection occurred on PN 90.

Figure 2.

Schematic diagram illustrating the preparation of crude synaptosomal and crude cytosolic samples. The dentate gyrus from both hemispheres was separated from the surrounding hippocampal tissue and homogenized in a glass Dounce homogenizer in homogenizing buffer. The resulting homogenate was then centrifuged at $1,000 \times g$ for 6 min at 4°C . The supernatant (S1) was then centrifuged again at $10,000 \times g$ for 15 min at 4°C . The resulting pellet (P2) was the crude synaptosomal fraction and the resulting supernatant (S2) was the crude cytosolic fraction. Both were stored at -80°C until use.

Figure 3.

Effect of prenatal ethanol and/or prenatal stress exposure on baseline cytoplasmic ARC protein levels. Representative bands from separate Western blots are presented above each corresponding data bar. Open bars: No Stress; filled bars: Stress. Data bars represent mean $\pm$ S.E.M. optical density normalized to the Sacc/No Stress control group. Asterisks denote significant differences between the Sacc/No Stress control group and all other prenatal exposure groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 4.

Effect of prenatal ethanol and/or prenatal stress exposure on baseline levels of AMPA receptor subunits. Representative bands from separate Western blots are presented above each corresponding data bar. **4A:** Basal synaptosomal GluA1 subunit expression. Open bars: No Stress; filled bars: Stress. Data bars represent mean $\pm$ S.E.M. optical density normalized to Sacc/No Stress. Asterisks denote significant main effect of prenatal ethanol exposure and significant differences between EtOH/Stress and Sacc/No Stress and Sacc/Stress; $*p < 0.05$, $**p < 0.01$. **4B:** Basal synaptosomal GluA2 subunit expression. Asterisk denotes significant difference between Sacc/No Stress and Sacc/Stress; $*p < 0.05$. **4C:** Sum total of synaptosomal GluA1:GluA2 expression under basal conditions. Filled bars: quantity of GluA1 localized in synaptosomal fraction; open bars: quantity of GluA2 localized in synaptosomal fraction. Data bars represent mean $\pm$ S.E.M. optical density normalized to Sacc/No Stress. Asterisks denote a significant difference in the total quantity of GluA1 and

GluA2 between the Saccharin and Ethanol groups within the prenatal stress exposure groups. The hatch symbol denotes a significant reduction in the quantity of GluA1 in the synaptosomal fraction in EtOH/Stress when compared to Sacc/No Stress control group; * $p < 0.05$, # $p < 0.05$.

Cytosolic ARC Expression Following TTTC

Figure 5.

Effect of prenatal ethanol and/or prenatal stress exposure on 2-trial Trace Conditioning (TTTC) -induced changes in cytoplasmic ARC protein levels. Representative bands from separate Western blots are presented above each corresponding data bar. Open bars: Unpaired Control (UPC); filled bars: TTTC. Data bars represent mean $\pm$ S.E.M. optical density normalized to Sacc/No Stress control group.

Figure 6.

Effect of prenatal ethanol and/or prenatal stress exposure on 2-trial Trace Conditioning (TTTC) -induced changes in AMPA receptor subunit expression. Representative bands from separate Western blots are presented above each corresponding data bar. **6A:** Synaptosomal GluA1 expression after TTTC. Open bars: Unpaired Control (UPC); filled bars: TTTC. Data bars represent the mean $\pm$ S.E.M., optical density normalized to Sacc/No Stress control group. Asterisks denote a significant difference between the UPC and TTTC conditions of the Sacc/No Stress group; $*p < 0.05$. **6B:** Synaptosomal GluA2 expression after TTTC. Open bars: UPC; filled bars: TTTC. Data bars represent mean $\pm$ S.E.M., optical density normalized to Sacc/No Stress control group. Asterisks denote significant differences between the UPC and TTTC conditions of the Sacc/No Stress group; $*p < 0.05$.