Published in final edited form as:

Alcohol Clin Exp Res. 2018 February ; 42(2): 295–305. doi:10.1111/acer.13574.

Prenatal alcohol exposure increases histamine H₃ receptor-mediated inhibition of glutamatergic neurotransmission in rat dentate gyrus

Rafael K. Varaschin, Nyika A. Allen, Martina J. Rosenberg, C. Fernando Valenzuela, and Daniel D. Savage

Department of Neurosciences, University of New Mexico School of Medicine, Albuquerque, New Mexico, 87131, USA

Abstract

Background—We have reported that prenatal alcohol exposure (PAE)-induced deficits in dentate gyrus (DG) long-term potentiation (LTP) and memory are ameliorated by the histamine H₃ receptor inverse agonist ABT-239. Curiously, ABT-239 did not enhance LTP or memory in control offspring. Here, we initiated an investigation of how PAE alters histaminergic neurotransmission in the DG and other brain regions employing combined radiohistochemical and electrophysiological approaches *in vitro* to examine histamine H₃ receptor number and function.

Methods—Long-Evans rat dams voluntarily consumed either a 0% or 5% ethanol solution four-hours each day throughout gestation. This pattern of drinking, which produces a mean peak maternal serum ethanol concentration of 60.8 ± 5.8 mg/dL, did not affect maternal weight gain, litter size or offspring birth weight.

Results—Radiohistochemical studies in adult offspring revealed that specific [³H]-A349821 binding to histamine H₃ receptors was not different in PAE rats compared to controls. However, H₃ receptor-mediated G_i/G_o protein-effector coupling, as measured by methimipip-stimulated [³⁵S]-GTPγS binding, was significantly increased in cerebral cortex, cerebellum and DG of PAE rats compared to control. A LIGAND analysis of detailed methimipip concentration-response curves in DG indicated that PAE significantly elevates receptor-effector coupling by a lower affinity H₃ receptor population without significantly altering the affinities of H₃ receptor subpopulations. In agreement with the [³⁵S]-GTPγS studies, a similar range of methimipip concentrations also inhibited electrically-evoked fEPSP responses and increased paired-pulse ratio, a measure of decreased glutamate release, to a significantly greater extent in DG slices from PAE rats than in controls.

Conclusion—These results suggest that a PAE-induced elevation in H₃ receptor-mediated inhibition of glutamate release from perforant path terminals as one mechanism contributing the LTP deficits previously observed in the DG of PAE rats, as well as providing a mechanistic basis for the efficacy of H₃ receptor inverse agonists for ameliorating these deficits.

Keywords

Fetal Alcohol Spectrum Disorder; Histamine H₃ Receptor; Methimipip; Glutamate; Dentate Gyrus

INTRODUCTION

It is well established that drinking during pregnancy can cause long-term neurobehavioral impairments in affected offspring (see review by Kodituwakku, 2009). Children with FASD exhibit a broad range of cognitive difficulties and behavioral-emotional problems (Mattson *et al.* 2013). Further, preclinical studies over the past thirty-five years have confirmed that prenatal or early postnatal exposure to alcohol produces a variety of behavioral deficits in affected offspring including behaviors homologous to behaviors altered in patients with FASD (for reviews, Riley, 1990; Kelley *et al.*, 2009; Schneider *et al.*, 2011; Patten *et al.*, 2014). Some of these behavioral deficits have been associated with functional damage to specific areas of the brain, such as the hippocampal formation, a brain region that appears to be particularly sensitive to perinatal ethanol exposure (Sutherland *et al.*, 1997; Perrone-Bizzozero *et al.*, 1998; Miki *et al.*, 2008; Samudio-Ruiz *et al.*, 2009; Titterness and Christie, 2012; Brady *et al.*, 2012). While multiple neurotransmitter systems are affected by exposure to alcohol during development (see review by Valenzuela *et al.*, 2012), efforts to link these prenatal alcohol exposure (PAE)-induced neurochemical alterations with specific functional and behavioral changes in PAE offspring has been limited.

We have reported subtle PAE-induced memory deficits in adult rat offspring whose mothers consumed moderate quantities of ethanol during pregnancy (Savage *et al.*, 2010). Analogous to patients with FASD, these deficits become more apparent with more challenging behavioral tasks (Sutherland *et al.* 2000; Weeber *et al.*, 2001, Savage *et al.*, 2002). “Baseline” physiological responses appear to be intact in our PAE rats while more complex, activity-dependent changes in synaptic plasticity are more sensitive to PAE (Sutherland *et al.*, 1997; Savage *et al.*, 1998; Costa *et al.*, 2000). A series of combined neurochemical and physiologic studies provided evidence suggesting that presynaptic mechanisms associated with activity-dependent increases in glutamate release from perforant path terminals in dentate gyrus are one mechanism contributing to the LTP deficits observed in our PAE rat offspring (Perrone-Bizzozero *et al.*, 1998; Savage *et al.*, 1998, 2002; Galindo *et al.*, 2004)

Collectively, these observations led us to search for agents that target presynaptic mechanisms regulating neurotransmitter release, which might ameliorate PAE-induced deficits in synaptic plasticity and learning. Agents acting as antagonists or inverse agonists at histamine H₃ receptors is one candidate receptor mechanism. These drugs act by blocking a presynaptic histamine H₃ receptor-mediated inhibition of the release of a variety of neurotransmitters including histamine (Arrang *et al.*, 1983), other monoamines (Schlicker *et al.*, 1988; 1989; 1993) and acetylcholine (Clapham & Kilpatrick, 1992). While activation of H₃ receptors also inhibits glutamate release (Brown & Haas, 1999; Garduno-Torres *et al.*, 2007), the question of whether H₃ receptor inverse agonists or antagonists can enhance glutamate release under basal or activity-dependent conditions has yet to be demonstrated.

Collectively, more than a dozen H₃ receptor antagonists or inverse agonists have been shown to have procognitive effects in a variety of animal models of learning and memory (see reviews by Haas *et al.*, 2008; Esbenschade *et al.*, 2008; Brioni *et al.*, 2011; Nikolic *et al.*, 2014).

Initial studies in our laboratory revealed that the H₃ receptor inverse agonist ABT-239 reverses PAE-induced deficits in LTP (Varaschin *et al.*, 2010) and learning (Savage *et al.*, 2010). Curiously, ABT-239 did not enhance either LTP or learning in control offspring. Further, treatment of control offspring with the selective H₃ receptor agonist methimipip (Kitbunnadaj *et al.*, 2005) mimicked the *in vivo* LTP deficit observed in saline-treated PAE rat offspring (Varaschin *et al.*, 2014). Taking these observations together, we hypothesized that PAE elevates histamine H₃ receptor-mediated inhibition of excitatory neurotransmission in the dentate gyrus. In an effort to begin investigating the mechanistic basis for the effects of PAE on histaminergic neurotransmission, we measured the number and function of H₃ receptors using a combination of radiohistochemical studies in various brain regions in conjunction with *in vitro* electrophysiological recordings in the same specific region of the dorsal dentate gyrus where we conducted *in vivo* electrophysiological recordings previously (Varaschin *et al.*, 2010, 2014). We speculated that PAE elevates H₃ receptor density in the dentate gyrus leading to heightened H₃ receptor-mediated inhibition of excitatory transmission at the perforant path – dentate granule cell synapse.

MATERIALS AND METHODS

Reagents

[³H]-A349821 was generously donated by Abbott Laboratories (Abbott Park, IL, USA). All other reagents were acquired from Sigma-Aldrich Corp (St. Louis, MO, USA) unless indicated otherwise in parenthetical text.

Voluntary Drinking Paradigm

All procedures involving the use of live rats were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee. Long-Evans rats (Harlan Industries, Indianapolis, IN, USA) housed at 22 °C on a reverse 12-hour dark/12-hour light schedule (lights on from 2100 to 0900 hours) and provided Harlan 2920 rodent chow and tap water *ad libitum*. The prenatal alcohol exposure and breeding procedures were the same as described previously (Savage *et al.*, 2010). After one-week of acclimation to the animal facility, all female breeders were single-housed and allowed to drink 5% ethanol in 0.066% saccharin in tap water for four hours each day from 1000 to 1400 hours. Daily four-hour ethanol consumption was monitored for at least two weeks and then the mean daily ethanol consumption determined for each female. Females whose mean daily ethanol consumption was greater than one standard deviation below the group mean, typically about 12–15% of the entire group, were removed from the study. The remainder of the females were assigned to either a saccharin control or 5% ethanol drinking group and matched such that the mean pre-pregnancy ethanol consumption by each group was similar. Subsequently, females were placed with proven male breeders until pregnant, as indicated by the presence of a vaginal plug.

Beginning on Gestational Day 1 (GD1), rat dams were provided saccharin water containing either 0% or 5% ethanol for four hours each day, from 1000 to 1400 hours. The volume of saccharin water provided to the control group was matched to the mean volume of saccharin water consumed by the ethanol group. Daily four-hour ethanol consumption was recorded for each dam through GD21, after which ethanol consumption was discontinued.

At birth, litters were culled to ten pups each. Offspring were weaned at 24 days of age and group-housed, two males or three female per cage until experimental use. Female offspring were used in all of the histochemical and biochemical procedures and male offspring were used in all of the electrophysiological procedures.

Serum Ethanol Assessment

Separate sets of twelve rat dams were used during two of the breeding rounds to assess the peak serum ethanol concentration. These dams were subjected to the same voluntary drinking paradigm as described above except that on GDs 13, 15 and 17, each dam was briefly anesthetized with isoflurane and a 100 μ L blood sample drawn from the tail vein 45 minutes after the introduction of the drinking tubes. The serum separated and the samples assayed for ethanol using a modification of the Lundquist assay (Lundquist, 1959). No offspring from the dams used in the serum ethanol assessment were used in the experimental procedures described below.

Histological Sectioning

Eight- to twelve-week-old offspring were sacrificed by rapid decapitation. Whole brains were dissected, frozen in isopentane chilled in a dry ice/methanol bath and stored in airtight containers at -80°C until sectioning. Twelve- μ m-thick microtome cryostat sections were collected in the sagittal plane corresponding to Lateral 1.40 mm in the Paxinos & Watson stereotaxic atlas of rat brain (Paxinos and Watson, 1998). This plane contained the same region of the superior blade of the dorsal dentate gyrus where recordings were made in the *in vitro* electrophysiological studies described below, as well as our previous *in vivo* electrophysiological studies (Varaschin et al, 2010, 2014). The sections were thaw-mounted onto pre-cleaned Superfrost-Plus[®] microscope slides (ColePalmer, Court Vernon Hills, IL, USA) and stored at -80°C in airtight containers until incubation. Characterization of [^3H]-A349821 binding (Figure 1) was conducted in a separate set of untreated control rats. Separate sets of control and PAE offspring were used for the: 1) “Two-point” saturation of [^3H]-A349821 binding study (Table 2), 2) brain region survey of methimipip-stimulated [^{35}S]-GTP γ S binding (Figures 2A & 2B) and 3) a more detailed methimipip dose-response study (Figure 3 and Table 3).

Specific [^3H]-A349821 Binding to Histamine H_3 Receptors

Histamine H_3 receptor density measures were performed using methods similar to those described by Witte et al., (2006). Tissue sections were incubated in buffer (150 mM Tris-HCl, 5 mM EDTA, 0.1% BSA, pH 7.4 at 25°C) with different concentrations of [^3H]-A349821 (Abbott Laboratories, specific activity = 54.0 Ci/mmol) for 60 minutes at 25°C in the absence (total binding) and presence of 10 μM thioperamide (non-specific binding). After incubation, sections were rinsed twice for 30 seconds each in ice-cold incubation

buffer, dipped in ice-cold distilled water, dried and placed in a vacuum desiccator overnight. Sections were then exposed to Kodak Biomax MR Film for four months and then the film developed in Kodak D-19 (1:1) and fixed. Microdensitometric measurement of [^3H]-A349821 binding was performed using Media Cybernetics Image Pro Plus[®] (Silver Spring, MD) on an Olympus BH-2 microscope at a total image magnification of 3.125X. An optical density standard curve, expressed in pCi/ $10^5 \mu\text{m}^2$, was established based on autoradiograms of tritium standards. Total and non-specific [^3H]-A349821 binding were measured in the superior blade of the dorsal dentate gyrus *stratum moleculare*, medial frontal cortex and the dorso-medial caudate nucleus. Specific [^3H]-A349821 binding, expressed as fmoles bound/ $10^5 \mu\text{m}^2$, was defined as the difference between total [^3H]-A349821 binding and non-specific [^3H]-A349821 binding.

Methimipip-stimulated [^{35}S]-GTP γ S Binding

Histamine H_3 agonist-stimulated receptor-effector coupling was performed using methods originally described by Sim et al. (1995). Tissue sections were pre-incubated for 10 minutes in incubation buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl_2 , 0.2 mM EGTA, 2 mM GDP and 100 nM DPCPX; pH 7.4 at 25 °C), and then incubated with 100 pM [^{35}S]-GTP γ S (Perkin Elmer, specific activity = 1250 Ci/mmol) for 90 minutes at 25 °C in the absence and presence of 10 μM unlabeled GTP γ S. In addition, sections were incubated with 2 μM methimipip in an initial study to obtain measures of H_3 receptor agonist-stimulated [^{35}S]-GTP γ S binding. In a subsequent more-detailed concentration-response study, sections were incubated with one of fifteen different concentrations of methimipip ranging from 0.1 nM to 5 μM . After incubation, sections were rinsed twice for 15 seconds each in ice-cold incubation buffer, dipped in ice-cold distilled water, dried and placed in a vacuum desiccator overnight. Sections were then exposed to Kodak Biomax MR Film for four days and then the film developed in Kodak D-19 (1:1) and fixed. Microdensitometric measurements of [^{35}S]-GTP γ S binding were performed similar to those described above except that the optical density standard curve was established based on autoradiograms of ^{14}C carbon standards. Binding measurements were performed over ten different brain regions. Basal [^{35}S]-GTP γ S binding was defined as the difference between binding in the absence and presence of 10 μM unlabeled GTP γ S. Methimipip-stimulated [^{35}S]-GTP γ S binding was defined as the difference between [^{35}S]-GTP γ S binding in the presence of methimipip and basal [^{35}S]-GTP γ S binding. Percent increase in agonist-stimulated [^{35}S]-GTP γ S binding was defined as methimipip-stimulated [^{35}S]-GTP γ S binding divided by basal [^{35}S]-GTP γ S binding.

Extracellular Slice Recording Procedures

Eight-week-old rat offspring were deeply anesthetized with ketamine (250 mg/kg i.p.), quickly decapitated and the brain removed. Coronal slices containing the dorsal dentate gyrus were obtained as described previously (Mameli *et al.*, 2005). Briefly, whole brain coronal slices (400 μm thick) were obtained using a vibrating microtome immersed in ice-cold cutting solution containing 3 mM KCl, 1.25 mM NaH_2PO_4 , 6 mM MgSO_4 , 26 mM NaHCO_3 , 0.2 mM CaCl_2 , 10 mM glucose, 220 mM sucrose, and 0.43 mM ketamine. Slices were then allowed to recover for 40 min at 32 °C in artificial cerebral-spinal fluid (ACSF) containing 126 mM NaCl, 2 mM KCl, 1.25 mM NaH_2PO_4 , 1 mM MgSO_4 , 26 mM NaHCO_3 , 2 mM CaCl_2 , 10 mM glucose and oxygenated with 95% O_2 /5% CO_2 . After

recovery, slices were maintained in oxygenated ACSF at room temperature until recording. Upon transfer to recording chambers, slices were perfused at a rate of 2 mL/min with 32 °C oxygenated ACSF containing different treatment conditions. Next, a concentric bipolar electrode (FHC, Bowdoin, ME) was placed in the medial portion of the superior blade of the dentate gyrus *stratum moleculare*, and a recording glass electrode (resistance ~3–5 Ω) filled with ACSF placed in the same layer, about 200 μ m lateral to the stimulating electrode. Electrically-evoked field excitatory post-synaptic potentials (fEPSPs) were triggered using a Master-8 stimulator and an Iso-flex stimulus isolator (AMPI, Jerusalem, Israel), and recorded using a Digitizer model 1440 and AxoPatch 200 amplifier (Molecular Devices, Sunnyvale, CA) with low-pass filter set at 2 kHz.

Input/output curves were generated using a stimulus intensity range of 0.05 to 1.2 mA to determine the stimulus intensity sufficient to elicit maximal fEPSP amplitude. All subsequent recordings were performed with a test stimulus intensity sufficient to elicit 40% of the maximal fEPSP response (ES₄₀). A paired-pulse stimulation of the medial perforant path protocol, consisting of two pulses of 75 μ s duration and 40 ms apart was used. Pulses were delivered every 30 seconds over ten minutes to establish a baseline response. Slices were then incubated for ten minutes with one of three concentrations of methimepip (0.3, 1.0, or 3.0 μ M). First pulse fEPSP data obtained over the last four minutes during each drug treatment condition was averaged as a single measure and normalized to the baseline fEPSP amplitude. Paired-pulse ratio (PPR) was measured as the ratio of the fEPSP amplitude elicited by the second pulse divided by the fEPSP amplitude elicited by the first pulse and averaged as a single value over the last four minutes of each treatment condition.

Study Design Issues and Statistical Analyses

For each type of study, no more than one offspring per litter was used in a given experiment to avoid the prospect of “litter effects”. Sample size determinations for these experiments indicated a minimum of n=6, based on past receptor binding studies and *in vitro* slice electrophysiological studies in our laboratories with an minimum expected difference in group means of 20%, an expected standard deviation of 10%, and a desired power of 0.8 at an alpha value of 0.05. Seven or eight pairs of subjects were used in all of the studies. There were no exclusion criteria and no data were excluded from the analyses. All microdensitometric measurements and slice incubations, except the electrophysiological recordings, were performed by investigators blinded to the prenatal treatment condition of the samples, and samples were analyzed randomly in pairs. Although the investigator was not blinded to treatment in the slice electrophysiology studies, the only *a priori* rejection criterion used in the study was the stability of the baseline fEPSP amplitude (a maximum of 20% drift was acceptable) and no slice met that criterion. Bias on the PPR and on the effect of methimepip on the EPSP amplitude is minimal because no decision to include/exclude data points were made after bath application of the drug.

All statistical procedures and graphical illustrations were performed using SigmaPlot® 11 (Systat Software Inc. San Jose, CA, USA). The effects of prenatal alcohol exposure on maternal weight gain, litter size, offspring birth weight (Table 1) were conducted using a Mann-Whitney Rank Sum test. Specific [³H]-A349821 binding (Table 2), methimepip-

stimulated [^{35}S]-GTP γ S binding at 2 μM (Figure 2B) and LIGAND kinetic estimates of methimepip-stimulated [^{35}S]-GTP γ S binding (Table 3) were analyzed using a two-tailed Student's t-test. Prenatal alcohol effects in the methimepip concentration-[^{35}S]-GTP γ S binding response study (Figure 3), input/output responses (Figure 4) and methimepip effects on first-pulse fEPSPs and PPRs (Figure 5) were analyzed using a repeated measures two-way ANOVAs. Post-hoc pairwise multiple comparisons were made using the Student-Newman-Keuls method. All data is expressed as the mean $\pm$ standard error of the mean (SEM) and a value of $p < 0.05$ was deemed as statistically significant.

RESULTS

Voluntary Drinking Paradigm

Rat dams consumed an average of 1.98 ± 0.07 g/kg/day of ethanol throughout gestation, a level of consumption that produced a mean serum ethanol concentration of 60.8 ± 5.8 mg/dL (Table 1) 45 minutes after the introduction of the drinking tubes, 30 minutes after the observed mean peak ethanol consumption time at 15 minutes (data not shown). This voluntary drinking paradigm did not affect maternal weight gain, litter size or offspring birth weight (Table 1).

Histamine H₃ Receptor Binding Studies

We first characterized specific [^3H]-A349821 binding to sagittal brain sections from untreated control rats. A349821 is a histamine H₃ receptor antagonist that binds with similar affinity to H₃ receptor isoforms (Witte et al., 2006). Figure 1A illustrates the distribution of total [^3H]-A349821 binding to histamine H₃ receptors in a sagittal section from an untreated rat brain. Histamine H₃ receptors exist in relatively low densities throughout the rat brain. Thus, extended periods of film exposure (four months) are required, due to the low receptor density coupled with the use of a tritiated radioligand. A longer exposure period increases the film background measure leading to a corresponding reduction in the signal to noise ratio and limiting the number of discrete brain regions where we could reproducibly quantitate [^3H]-A349821 binding. Nevertheless, specific [^3H]-A349821 binding was heterogeneously distributed across the brain with relatively higher densities occurring in the caudate nucleus, intermediate levels in the middle and deep layers of the cerebral cortex and lower levels in the dentate gyrus (Figure 1A). As it was difficult to visualize specific [^3H]-A349821 binding in the dentate gyrus at the lower radioligand concentrations used in the initial saturation of binding study, we used measurements of specific [^3H]-A349821 binding in the medial frontal cortex to characterize the binding reaction (Figure 1B and 1C). Specific [^3H]-A-349821 binding in medial frontal cortex of untreated control rats suggested that [^3H]-A349821 bound to a single non-interacting population of binding sites with an apparent affinity constant of 11.7 nM and a B_{max} of $0.324 \text{ fmoles}/10^5 \mu\text{m}^2$ (Figure 1C).

We next examined whether PAE elevated the density of histamine H₃ receptors in the dentate gyrus, as well as medial frontal cortex and the caudate nucleus. The quantity of donated radioligand remaining was not sufficient to conduct a full saturation of specific [^3H]-A349821 receptor binding study in control and PAE rats. Thus, sagittal sections from both control and PAE rat offspring were incubated in the presence of either a “near K_d ” (9 nM) or

“near-saturating” (33.6 nM) concentration of [^3H]-A349821, and H_3 receptor binding site densities measured in the dentate gyrus, medial frontal cortex and caudate nucleus. No differences in specific [^3H]-A349821 binding were noted between controls and PAE offspring at either radioligand incubation concentration in any of the three brain regions analyzed (Table 2).

Histamine H_3 Receptor-Effector Coupling Studies

The specific [^3H]-A349821 binding studies suggested that PAE does not increase histamine H_3 receptor density (Table 2). We next examined whether PAE affected histamine H_3 receptor-effector coupling by incubating brain sections with [^{35}S]-GTP γS in the presence of the H_3 agonist methimipip. Methimipip-stimulated [^{35}S]-GTP γS binding displayed a qualitatively similar heterogeneous pattern of distribution in sagittal sections of rat brain (Figure 2A) as total [^3H]-A349821 binding (Figure 1A). However, the agonist-stimulated signal coupled with the higher specific activity of [^{35}S]-GTP γS yielded greater autoradiographic contrast, allowing the quantitation of histamine H_3 receptor activity in additional regions, such as the cerebellar *stratum moleculare*, that were not readily discernable in autoradiograms of specific [^3H]-A349821 binding.

In an initial study in control and PAE rats, a near-maximally effective concentration of methimipip (2 μM) was used to measure histamine H_3 agonist-stimulated [^{35}S]-GTP γS binding in ten different brain regions. Basal [^{35}S]-GTP γS binding was not different between the two prenatal treatment groups in any of the brain regions measured (data not shown). In control rats, methimipip-stimulated [^{35}S]-GTP γS binding was highest in medial frontal cortex, followed by intermediate levels in parietal cortex, nucleus accumbens, and caudate nucleus, and lower levels in dentate gyrus, cerebellum and the superior and inferior colliculi (Figure 2B). In contrast to specific [^3H]-A349821 binding, methimipip-stimulated [^{35}S]-GTP γS binding was significantly elevated in the dentate gyrus, cerebellum and cortical regions of PAE rats compared to controls. H_3 agonist-stimulated [^{35}S]-GTP γS binding was not different in other subcortical brain regions.

A subsequent more-detailed agonist dose-response study, focused on the dentate gyrus, revealed that methimipip-stimulated [^{35}S]-GTP γS binding increased above baseline over a roughly four orders of magnitude increase in methimipip concentration (Figure 3). A two-way ANOVA revealed significant effects of methimipip ($F(1,15) = 90.3$, $p < 0.001$) and prenatal treatment group ($F(1,15) = 31.4$, $p < 0.001$) and no interaction between factors. At the apparent near-maximally effective agonist concentration of 5 μM methimipip, [^{35}S]-GTP γS binding was 82% greater than basal [^{35}S]-GTP γS binding in control offspring and 148% above basal binding in PAE offspring. A Student-Newman-Keuls pairwise multiple comparison analysis revealed that methimipip-stimulated [^{35}S]-GTP γS binding was significantly greater in PAE offspring compared to controls at methimipip concentrations at and above 0.1 μM (Figure 3). A kinetic analysis of the H_3 agonist dose-response data using LIGAND indicated the presence of two agonist-sensitive subpopulations of histamine H_3 receptors (Table 3). In control rats, the higher ($k_{\text{D}1}$) and lower ($k_{\text{D}2}$) affinity H_3 receptor subpopulations each accounted for about one-half of the maximal agonist response, with apparent affinity constants of 0.868 and 422 nM respectively. In PAE rats, the apparent

affinity constants were not significantly different from controls, nor was the agonist response to the higher affinity receptor population (Re_{max1}) different. In contrast, the agonist response at the lower affinity population (Re_{max2}) was significantly greater in PAE rats compared to controls (Table 3).

Effects of Methimepip on Glutamatergic Synaptic Transmission in the Dentate Gyrus

In the final set of experiments, we examined whether there was a physiological correlate to the observed PAE-induced increase in H_3 receptor-effector coupling by conducting extracellular recording of granule cell responses to perforant path-evoked stimulation in slices of dentate gyrus. The recordings were performed in the same region of the dentate gyrus *stratum moleculare* as studied in the radiohistochemical experiments. Increased electrical stimulation of perforant path fibers in the medial layer of the dentate gyrus *stratum moleculare* resulted in increased fEPSP responses. However, as was observed previously in *in vivo* electrophysiological studies in control and PAE rats (Varaschin et al, 2010) PAE did not affect input/output responses dentate granule cells in slices (Figure 4).

The effects of methimepip and PAE on electrically-evoked fEPSP responses and on the PPR measure of glutamate release are depicted in Figure 5. Perfusing control slices with increasing concentrations of methimepip (0.3 – 3 μ M) resulted in a modest decrease in fEPSP responses (Figure 5B). In contrast, methimepip produced greater inhibition of fEPSP responses in slices from PAE rat offspring. A two-way ANOVA indicated significant effects of both methimepip ($F(1,3) = 8.08$, $p < 0.001$) and prenatal treatment group ($F(1,3) = 5.91$, $p = 0.019$), but no significant interaction between factors. Post-hoc analysis revealed a significant difference between the control and PAE group only at the 3 μ M concentration of methimepip (Figure 5B). Methimepip also dose-dependently increased the paired-pulse ratio (PPR) measure of glutamate release to a greater extent in PAE rats compared to controls (Figure 5C). Increasing the PPR is an index of a reduction in glutamate release probability. A two-way ANOVA indicated significant effects of both methimepip ($F(1,3) = 16.3$, $p < 0.001$) and prenatal treatment group ($F(1,3) = 10.4$, $p < 0.001$) but no significant interaction between factors. A post-hoc analysis revealed a significant difference between the control and PAE group at both the 1 μ M and 3 μ M concentrations of methimepip (Figure 5C).

DISCUSSION

The salient findings in this study are that prenatal exposure to moderate levels of alcohol increased methimepip-stimulated H_3 receptor-effector coupling in dentate gyrus as well as enhanced methimepip's inhibitory effects on first pulse fEPSPs and glutamate release. Further, the concentrations of methimepip that reduced fEPSP responses (Figure 5B) and heightened the PPR (Figure 5C) were similar to the methimepip concentrations that enhanced [35 S]-GTP γ S binding (Figure 3) measured in the same region within the superior blade of the dentate gyrus where the electrophysiological studies were performed. That methimepip-stimulated [35 S]-GTP γ S binding was also elevated in a variety of cortical regions and cerebellum of PAE rats compared to control (Figure 2B) suggests that PAE-induced elevation in histamine H_3 receptor-mediated inhibition of glutamatergic neurotransmission may occur across large areas of the higher brain.

The mechanistic basis for elevated H₃ receptor-effector coupling in PAE (Figure 2B) is currently unknown. Specific [³H]-A349821 binding data was not different between prenatal treatment groups (Table 2), suggesting that the total density of histamine H₃ receptors in PAE rat brain is similar to controls (Table 2) and thus, not the basis for differential agonist-mediated responses. One possible explanation for heightened H₃ receptor-effector coupling could be a PAE-induced alteration in the differential expression of H₃ receptor isoforms expressed by entorhinal cortical neurons projecting to the dentate gyrus. At least three functional isoforms of histamine H₃ receptors are expressed in rats (Morisset et al., 2001). These isoforms, which originate from alternative splicing of the H₃ receptor gene, differ primarily in the length of the amino acid sequence within the third cytoplasmic loop of this transmembrane receptor protein. The truncated isoforms, identified in rat as the rH_{3B} and rH_{3C} isoforms, exhibit higher agonist affinity and lower intrinsic activity in comparison to the longer rH_{3A} isoform which has lower agonist affinity and higher intrinsic activity (Bongers et al., 2007; Drutel et al., 2001). One reasoned speculation is that PAE may have shifted the predominant H₃ receptor isoform expressed in entorhinal cortical neurons from the higher affinity less intrinsically active rH_{3B} or rH_{3C} isoforms to the lower affinity more intrinsically active rH_{3A} isoform. Whereas there are, currently, no receptor subtype-selective H₃ receptor drugs or antibodies to directly examine this question, one alternative approach was to conduct a detailed H₃ agonist dose-response study in control and PAE rats. While there was a small shift in the proportional agonist response between the higher and lower affinity receptor populations in PAE rats compared to controls (Table 3) which is consistent with speculation about a differential shift in the expression of H₃ receptor isoforms, it is unclear whether a small shift in the differential expression of H₃ receptor isoforms alone could account for the differential agonist responses (Figure 3, Figures 5B and 5C) observed in PAE rats.

One mechanism for increased intrinsic activity of the receptor-effector response could be an increase in the quantity of G_{i/o} protein levels in the perforant path nerve terminals. However, the observation that basal [³⁵S]-GTPγS binding was not different between prenatal treatment groups (data not shown) does not support this idea. Another explanation could relate to the involvement of intracellular mechanisms that regulate G-protein coupling of metabotropic receptors to their effector mechanisms. While virtually nothing is known about these regulatory processes with respect to H₃ receptors, there is evidence that various protein kinases and other factors regulate the coupling of another G_{i/o} protein-coupled receptor, namely the mGluR₇ receptor (Sorensen et al., 2002), which also regulates the release of glutamate (Pelkey et al., 2005; Zhang et al., 2008). This is an intriguing prospect relative to the differential receptor-effector coupling response in PAE rats given that some of these regulatory mechanisms target the third cytoplasmic loop of the mGluR₇ receptor.

Whatever the mechanistic basis for elevated H₃ receptor-effector coupling in PAE rats, this alteration provides one explanation for methimipip's greater inhibitory effect on fEPSP responses and glutamate release (Figure 5B and 5C) in PAE rats. The possible mechanisms by which histamine H₃ receptors inhibit glutamate release can be inferred based on evidence from studies of other presynaptic G_{i/o}-protein coupled receptors (reviewed by Betke et al., 2012). Upon activation, these G-proteins dissociate into G_{i/o} α and βγ subunits. The activated G_{i/o} α-subunit inhibits adenylyl-cyclase activity, leading to reduced cAMP levels

and, consequentially, reduced protein kinase A (PKA) activity. As presynaptic voltage-gated calcium channels are a substrate for PKA, reduced phosphorylation of these channels could decrease neurotransmitter release. Additionally, the activated G_i/G_o $\beta\gamma$ -subunit directly inhibits voltage-gated calcium channels at axon terminals as well as soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein, leading to reduced synaptic vesicle exocytosis, further contributing to decreased neurotransmitter release. Histamine inhibits dentate gyrus granule cell excitability and glutamate release, probably by a direct blockade of voltage gated calcium channels by $\beta\gamma$ subunits of $G_{i/o}$ protein (Brown and Haas, 1999; Brown and Reymann, 1996). Here, it remains to be investigated whether or not PAE increases H_3 receptor mediated inhibition of adenylate cyclase and/or reduces voltage-gated calcium channel conductance as potential mechanisms for the observed differential effect on glutamate release.

Given prior studies demonstrating the inhibitory effects of H_3 receptor agonists on glutamate release and dentate granule cell responses both *in vitro* (Brown and Reymann, 1996) and *in vivo* (Managhan-Vaughn et al., 1998; Varaschin et al., 2014), one somewhat unexpected result in the present study was the observation that methimip produced only modest inhibition of fEPSP responses or glutamate release in control offspring (Figure 5B and 5C). Differences in slice electrophysiological approaches between laboratories may, in part, account for this apparent discrepancy. In the present study, one putative explanation for the different degrees of responses to methimip by twelve- μ m-thick sections used in radiohistochemical studies compared to 400- μ m-thick slices used in physiological studies may be that higher agonist concentrations are required to penetrate slices to elicit responses of similar magnitude. Thus, it is possible that a greater degree of methimip inhibition of fEPSP responses and glutamate release in control slices may have been observed had higher agonist concentrations been employed in this study. Nevertheless, the results indicate that PAE rats were more responsive to the inhibitory effects of methimip than controls.

In summary, these *in vitro* studies provide the first report of a heightened histamine H_3 receptor-mediated receptor-effector coupling associated with an inhibition of glutamate release from entorhinal cortical projections to the dorsal dentate gyrus of PAE rats compared to control offspring. This elevated inhibitory drive provides one mechanistic explanation for our prior observations of deficits in dentate gyrus LTP (Varaschin et al., 2010). However, given the complexity of the intrinsic and extrinsic regulation of dentate granule cell responsiveness, studies employing microinjections of a histamine H_3 inverse agonist directly into the dentate gyrus will be required to further substantiate this interpretation. In addition, other putative mechanisms of H_3 receptor inverse agonist/antagonist action in the dentate gyrus remain to be investigated. For example, the inhibition of H_3 receptors on cholinergic nerve terminals facilitates acetylcholine release (Fox et al., 2005), which, in turn, could facilitate glutamatergic neurotransmission. In addition, H_3 receptor antagonist-mediated inhibition of H_3 autoreceptors located on histaminergic nerve terminals promotes histamine release (Arrang et al., 1983), which may facilitate excitation of glutamatergic neurons mediated via histamine postsynaptic H_1 and H_2 receptors (Haas, 1984; Selbach et al., 1997; Manahan-Vaughan et al., 1998). Further, histamine has been reported to have positive allosteric effects at the spermidine site on NMDA receptors (i.e.: Bekkers, 1993; Vorobjev et al., 1993). The extent to which each of these other mechanisms of H_3 receptor inverse

agonist/antagonist action may have contributed to an amelioration of a synaptic plasticity deficit in PAE offspring (Varaschin et al, 2010) is not known, but it is likely that the manner by which H₃ receptor inverse agonists/antagonists enhance synaptic transmission at glutamatergic synapses in the central nervous system is complex, may be dose-dependent, and may also depend on level of arousal (Luo and Leung, 2010).

Acknowledgments

This work was supported by NIAAA grants 1 P20 AA017068, 1 RO1 AA019884 and 1 P50 AA022534.

The authors have no conflict of interest related to the information communicated in this report.

The authors wish to thank Dr. Marion Cowart, Dr. Timothy Esbenshade, Dr. Tiffany Garrison, and Dr. Thomas Miller of Abbott Laboratories for their technical advice on the use of the [³H]-A349821 autoradiography methods. The authors would also like to thank Dr. Stefano Zucca and Ms. Aya Wadleigh in the Department of Neurosciences at the University of New Mexico for their advice on the electrophysiological studies of dentate gyrus

ABBREVIATIONS

FASD	Fetal Alcohol Spectrum Disorder
fEPSP	field Excitatory Post-Synaptic Potential
LTP	Long-term potentiation
PAE	Prenatal Alcohol Exposure
PPR	Paired-pulse ratio
DG	Dentate gyrus

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FIGURE 1.

Characterization of [³H]-A349821 binding in control offspring. 1A: Representative autoradiogram of total [³H]-A349821 binding in a sagittal section from an untreated control rat. The three brain regions where binding measurements were made, as denoted by black dotted lines, were the superior blade of the dentate gyrus *stratum moleculare* (DGS) the dorsal medial caudate nucleus (CAU) and the medial frontal cortex (MFC). The black bar in lower right corner denotes a distance of 1 mm. 1B: Saturation of specific [³H]-A349821 binding in medial frontal cortex of control offspring. Data points are the mean $\pm$ SEM of binding expressed as femtomoles/ $10^5 \mu\text{m}^2$ at five different concentrations of radioligand in six untreated control rat offspring. 1C: Scatchard transformation of the saturation of specific [³H]-A349821 binding from Figure 1B.

FIGURE 2.

Impact of PAE on H_3 receptor agonist-stimulated receptor-effector coupling in various brain regions of adult rat offspring. 2A: Representative autoradiogram of total $[^{35}S]$ -GTP γ S binding in the presence of 2 μ M methimipip in a sagittal section from a saccharin control rat. The ten brain regions where binding measurements were made, as denoted by white dotted lines, were the medial frontal cortex (MFC), nucleus accumbens core (NAC), dorsal medial caudate nucleus (CAU), deep layer- and middle layer of the medial parietal cortex (DPC, MPC), superior and inferior blades of the dentate gyrus *stratum moleculare* (DGS, DGI) superior colliculus (SC), inferior colliculus (IC) and cerebellar *stratum moleculare* (CB). The black bar in the lower right corner denotes of 1 mm. 2B: Effect of PAE on methimipip-stimulated $[^{35}S]$ -GTP γ S binding. Data bars represent the mean $\pm$ SEM of 7 pairs of control and PAE rats. Asterisks denote data significantly greater than control (* $p < 0.05$; ** - $p < 0.005$).

FIGURE 3.

Effect of increasing concentrations of methimipip on [^{35}S]-GTP γ S binding in the superior blade of the dorsal dentate gyrus *stratum moleculare* in control and PAE rats. Data points represent the mean $\pm$ the SEM, expressed as percent of basal [^{35}S]-GTP γ S binding, in eight pairs of control and PAE rats. Asterisks denote methimipip concentrations at which methimipip-stimulated [^{35}S]-GTP γ S binding was significantly greater in PAE rats compared to controls (* - $p < 0.05$).

FIGURE 4.

Effects of prenatal alcohol exposure (PAE) on baseline field excitatory postsynaptic potentials (fEPSPs) recorded from dorsal dentate gyrus granule cells. 4A: Representative traces illustrating fEPSP responses from a control rat to single pulses of 0.1 to 1.2 mA stimulus intensity. 4B: A summary of fEPSP response data in control and PAE rats. Data points represent the mean $\pm$ SEM fEPSP response amplitude, expressed in mV, from seven pairs of control and PAE rats.

FIGURE 5.

Effects of prenatal alcohol exposure (PAE) on histamine H_3 receptor-mediated inhibition of fEPSP responses and paired-pulse ratio. 5A: Representative fEPSP traces evoked by pairs of pulses given at a 40 millisecond interpulse interval are shown at zero and 3 μ M methimipip for control and PAE rats. 5B: Effect of PAE on methimipip inhibition of first-pulse fEPSP amplitude responses. Data bars represent the mean $\pm$ SEM first-pulse fEPSP amplitude, expressed as percent of saccharin control baseline responses, from seven pairs of control and PAE rats. Asterisks denote significantly greater inhibition in PAE rats compared to controls (* - $p < 0.05$). 5C: Effects of PAE on methimipip enhancement of the paired-pulse ratio. Data bars represent the mean $\pm$ SEM paired-pulse ratio, expressed as the second fEPSP pulse amplitude/first fEPSP pulse amplitude, in seven pairs of control and PAE rats. Asterisks denote a significantly higher PPR, reflecting a lower probability of glutamate release in PAE rats compared to controls (* - $p < 0.05$).

Table 1

Effects of daily four-hour consumption of 5% ethanol on female rat dams and their offspring

	Saccharin Control	Prenatal Alcohol-exposed
Daily Four-hour 5% Ethanol Consumption	NA	1.98 ± 0.07 ^a (44)
Maternal Serum Ethanol Concentration	NA	60.8 ± 5.8 ^b (24)
Maternal Weight Gain During Pregnancy	112 ± 5 ^c (41)	100 ± 4 (44)
Litter Size	11.4 ± 0.4 ^d (41)	10.8 ± 0.4 (44)
Offspring Birth Weight	9.90 ± 0.52 ^e (41)	9.00 ± 0.29 (44)

^aMean ± S.E.M. grams ethanol consumed/kg body weight/day^bMean ± S.E.M. mg ethanol/dL serum, 45 minutes after introduction of the drinking tubes^cMean ± S.E.M. grams increase in body weight from GD 1 through GD 21^dMean ± S.E.M. number of live births/litter^eMean ± S.E.M. grams pup birth weight

NA - Not applicable

(n) - Group sample size

TABLE 2

Impact of PAE on specific [^3H]-A349821 binding at half-maximally and three-quarter-saturating concentrations of radioligand in three brain regions in adult rat. Data values are the mean $\pm$ (SEM), expressed as femtomoles bound/ $10^5 \mu\text{m}^2$, in seven pairs of control and PAE rat offspring.

[^3H]-A349821 (nM): BRAIN REGION	9.0 nM		33.6 nM	
	Saccharin Control	Prenatal Alcohol-exposed	Saccharin Control	Prenatal Alcohol-exposed
Dentate Gyrus (Superior blade <i>Stratum moleculare</i>)	0.080 (0.007)	0.082 (0.006)	0.229 (0.029)	0.228 (0.024)
Medial Frontal Cortex (Cingulate Area 3)	0.143 (0.014)	0.149 (0.010)	0.253 (0.034)	0.300 (0.027)
Caudate Nucleus (Dorso-medial)	0.231 (0.019)	0.237 (0.013)	0.453 (0.039)	0.457 (0.035)

TABLE 3

Impact of PAE on the kinetic characteristics of methimipip-stimulated [^{35}S]-GTP γ S binding in the superior blade of dorsal dentate gyrus. Data values are the mean $\pm$ (SEM), expressed as femtomoles bound/ $10^5 \mu\text{m}^2$, in seven pairs of control and PAE rat offspring.

Dentate Gyrus	$\text{Re}_{\text{max}}1^a$	K_d1^b	$\text{Re}_{\text{max}}2^c$	K_d2^d
Saccharin Control	48.4 (5.6)	0.868 (0.188)	46.3 (5.9)	422 (116)
Prenatal Alcohol Exposed	73.5 (12.0)	0.834 (0.241)	79.2* (11.1)	643 (239)

^a Estimated maximal response to the higher affinity H₃ receptor population (% of basal response).

^b Estimated affinity constant for methimipip stimulation of the high affinity H₃ receptors (μM).

^c Estimated maximal response to the lower affinity H₃ receptor population (% of basal response).

^d Estimated affinity constant for methimipip-stimulation of the lower affinity H₃ receptors (μM).

* denotes data significantly greater than the saccharin control group ($p=0.023$)