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Prenatal exposure to ethanol stimulates hypothalamic CCR2 chemokine receptor system: Possible relation to increased density of orexigenic peptide neurons and ethanol drinking in adolescent offspring

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Abstract

Clinical and animal studies indicate that maternal consumption of ethanol during pregnancy increases alcohol drinking in the offspring. Possible underlying mechanisms may involve orexigenic peptides, which are stimulated by prenatal ethanol exposure and themselves promote drinking. Building on evidence that ethanol stimulates neuroimmune factors such as the chemokine CCL2 that in adult rats is shown to colocalize with the orexigenic peptide, melanin-concentrating hormone (MCH) in the lateral hypothalamus (LH), the present study sought to investigate the possibility that CCL2 or its receptor CCR2 in LH are stimulated by prenatal ethanol exposure, perhaps specifically within MCH neurons. Our paradigm of intraoral administration of ethanol to pregnant rats, at low-to-moderate doses (1 or 3 g/kg/day) during peak hypothalamic neurogenesis, caused in adolescent male offspring two-fold increase in drinking of and preference for ethanol and reinstatement of ethanol drinking in a two-bottle choice paradigm under an intermittent access schedule. This effect of prenatal ethanol exposure was associated with an increased expression of MCH and density of MCH⁺ neurons in LH of preadolescent offspring. Whereas CCL2⁺ cells at this age were low in density and unaffected by ethanol, CCR2⁺ cells were dense in LH and increased by prenatal ethanol, with a large percentage (83–87%) identified as neurons and found to colocalize MCH. Prenatal ethanol also stimulated the genesis of CCR2⁺ and MCH⁺ neurons in the embryo, which co-labeled the proliferation marker, BrdU. Ethanol also increased the genesis and density of neurons that co-expressed CCR2 and MCH in LH, with triple-labeled CCR2⁺/MCH⁺/BrdU⁺ neurons that were absent in control rats accounting for 35% of newly generated neurons in ethanol-exposed rats. With both the chemokine and MCH systems believed to promote ethanol consumption, this greater density of CCR2⁺/MCH⁺ neurons in the LH of preadolescent rats suggests that these systems function together in promoting alcohol drinking during adolescence.

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Keywords

prenatal ethanol; lateral hypothalamus; melanin-concentrating hormone; CCL2; CCR2; neurogenesis

INTRODUCTION

Preference for and overconsumption of alcohol, in addition to being genetically determined, are affected by early environmental factors, including gestational exposure to ethanol that produces profound disturbances in growth and development, with long-term behavioral consequences. In humans, maternal drinking during pregnancy increases the risk for development of alcohol use and abuse and also for early onset of alcohol-related disorders (Baer et al., 2003, Alati et al., 2006, Malone et al., 2010). Laboratory animals with gestational exposure to ethanol also exhibit an increase in ethanol preference and consumption (Chotro et al., 2007, Youngentob and Glendinning, 2009, Diaz-Cenzano and Chotro, 2010), in addition to other behavioral disturbances associated with drug abuse (Carneiro et al., 2005, Stettner et al., 2011, Cullen et al., 2013). While these clinical and preclinical studies provide clear evidence for a stimulatory effect of prenatal ethanol exposure on later alcohol consumption, the neurochemical and molecular mechanisms underlying this behavioral phenomenon still remain to be characterized.

There is evidence for the involvement of the neurotransmitters, dopamine, serotonin, and gamma-aminobutyric acid, which are known to have a role in mediating ethanol acquisition, consumption, reward, reinforcement and tolerance (McBride et al., 1990, Koob et al., 1998, Vengeliene et al., 2008, Tabakoff et al., 2009) and to be stimulated by ethanol in adult animals (Gatto et al., 1994, Mihic et al., 1995, Carta et al., 2004, Jonsson et al., 2014) and altered by prenatal exposure to moderate or high doses of ethanol (Chotro et al., 2007). In addition, considerable clinical and preclinical studies support a role for hypothalamic orexigenic peptides in alcohol abuse (DiLeone et al., 2003, Karlsson et al., 2012, Li et al., 2014), including melanin-concentrating hormone (MCH) and orexin (OX) expressed in the lateral hypothalamus (LH). In adult animals, consumption or injection of ethanol stimulates endogenous expression and synthesis of both MCH and OX (Morganstern et al., 2010a, Barson et al., 2014), and central injection of these peptides, in turn, increases the drinking of ethanol, while the opposite effect is induced by their receptor antagonists (Richards et al., 2008, Cippitelli et al., 2010, Shoblock et al., 2011). Moreover, in a recent report (Chang et al., 2012), we demonstrated that exposure to ethanol during gestation, at low-to-moderate doses (1 or 3 g/kg/day) that elevate blood ethanol concentration (BEC) to <100 mg/dL, markedly stimulates neurogenesis and increases the density and long-term expression of the orexigenic peptides, OX in the LH as well as galanin and enkephalin in the hypothalamic paraventricular nucleus (Chang et al., 2012). Although yet to be investigated, recent studies of MCH showing its role in mediating the rewarding and motivating properties of ethanol (DiLeone et al., 2003, Duncan et al., 2005, Kim et al., 2012) suggest that neurons expressing this peptide are likely to be similarly affected by gestational ethanol and, in turn, to contribute significantly to the phenomenon of excess alcohol drinking.

In seeking information on the molecular mechanisms that mediate this stimulatory effect of ethanol on the development of these peptidergic neurons and their long-term expression, we were particularly interested in the evidence that relates MCH to the neuroimmune system, showing this peptide to coexist with the chemokine, CCL2, in the LH (Banisadr et al., 2005). Ethanol is known to affect neuroimmune factors, cytokines and chemokines, which in turn are found to have profound effects on neuronal development and migration (Li and Ransohoff, 2008, Boulanger, 2009, Williamson and Bilbo, 2013). Excessive use of alcohol markedly increases these factors in the periphery and brain, and their levels are positively related to the craving for alcohol (He and Crews, 2008, Qin et al., 2008, Crews et al., 2011). There is strong clinical evidence focusing attention on CCL2, with alcoholics exhibiting a marked increase in its protein levels in the blood and cerebrospinal fluid (Umhau et al., 2014) and also in limbic brain regions (He and Crews, 2008). In addition, acute or chronic administration of ethanol in adult animals increases CCL2 mRNA and protein in the periphery and extra-hypothalamic brain areas (Kane et al., 2014, Pascual et al., 2015), with effects after repetitive exposure persisting for days (Qin et al., 2008). Similar changes in CCL2 mRNA have been observed with chronic neonatal ethanol administration (Drew et al., 2015), and there is one study of the CCL2 receptor, CCR2, showing that it is also increased in peripheral tissue by ethanol (Wang et al., 2012). A possible function of the CCL2/CCR2 signaling pathway in promoting ethanol drinking is suggested by evidence that deletion of the CCL2 or CCR2 gene markedly reduces the intake of ethanol (Blednov et al., 2005).

Together, these findings led us to investigate in animals prenatally exposed to ethanol a possible relationship of the hypothalamic CCL2/CCR2 system to the development of MCH-expressing neurons in the LH and also to the ethanol-induced increase in later consumption of alcohol. In these experiments, we used our previously published model of prenatal ethanol exposure which was effective in stimulating the genesis and expression of OX neurons in the LH (Chang et al., 2012). This animal model involves intraoral administration to the dam of ethanol at low-to-moderate doses (1 or 3 g/kg/day) for six days, from embryonic day 10 (E10) to E15 when hypothalamic neurogenesis occurs (Chang et al., 2012). Our first goal in the present study was to determine whether this model involving maternal ethanol administration does, in fact, induce an increase in the consumption of and preference for ethanol in the offspring during adolescence. We next examined in preadolescent rats whether prenatal ethanol can stimulate the expression and density of MCH neurons in the LH and also of CCL2 or CCR2 neurons in the same hypothalamic area. To understand the relationship between this chemokine system and MCH, we then investigated whether CCL2 or CCR2 co-localizes with MCH in these preadolescent rats and, if so, whether the genesis of these co-localizing neurons is affected by ethanol *in utero*. Positive results relating these systems would suggest a possible involvement of the CCL2/CCR2 system in the neurochemical and behavioral effects induced by a relatively low dose of ethanol.

METHODS AND PROCEDURES

Animals

Time-pregnant, Sprague-Dawley rats (220–240g) (Charles River Breeding Laboratories (Hartford, CT) arrived on embryonic day 5 (E5) and were individually housed in plastic cages, in a fully accredited AAALAC facility (22°C, 12:12-h light-dark cycle with lights off at 2 pm), according to institutionally approved protocols as specified in the NIH Guide to the Care and Use of Laboratory Animals and with approval of The Rockefeller University Animal Care and Use Committee. Rats were acclimated to laboratory conditions until E10, when either a 1 g/kg/day (30%) ethanol solution, 3 g/kg/day (40%) ethanol solution, or a control solution of maltose-dextrin (made isocaloric to 35% ethanol solution) were intraorally administered (Chang et al., 2012). The daily dose of ethanol was split in half, with all rats gavaged two times daily, 4 h before the end of the light cycle and 4 h into the dark cycle, until E15. Blood ethanol concentration, measured 2 h after gavage, yielded 51 ± 4.8 mg/dL in the 1 g/kg/day ethanol group and 90 ± 9.5 mg/dL in the 3 g/kg/day ethanol group, consistent with previous reports (Qiang et al., 2002, Chang et al., 2013). Rodent chow (LabDiet Rodent Chow 5001, St. Louis, MO) and water were available *ad libitum*. Food intake was measured 3 times per week, and body weight was recorded weekly. There were no differences between control and ethanol-treated dams in their body weight (245–265 g) or chow intake (62–78 kcal/day). At birth, on postnatal day 0 (P0), litters were culled to n=8. Litters of the ethanol and control dams were similar in terms of size (from 9–15), body weight (5.7–6.8 g), and male/female ratio (1.1/1.0), with no spontaneous abortions observed in any of the groups. Only male offspring were tested, with 1 male pup taken from each litter and the number of rats/group (n=5–10) equal to the number of litters. No differences in body weight were evident between the ethanol vs control groups of offspring at P25 (41–53 vs 38–60 g) or at P40 (150–168 vs 142–160 g). At these ages, the whole brain or dissected brain tissue was collected for further analyses, all of which were performed by an evaluator blind to the experimental conditions.

Ethanol Intake and blood ethanol concentration

Male offspring (P40) of dams exposed to ethanol (1 or 3 g/kg/day) or control solution were trained to voluntarily drink ethanol using the 20% intermittent-access paradigm, as previously described (Simms et al., 2008, Barson et al., 2015). During the three weeks of access, ethanol, water and chow intake were recorded. On the last day of the testing period, blood was collected from the tail vein 30 min after the daily ethanol presentation, and blood ethanol concentration (BEC) was measured (Analoz GM7 Alcohol Analyzer, Lunenburg, MA, USA).

Quantitative real-time PCR

Offspring were sacrificed at P25, and the LH was dissected and total RNA from each microdissected sample was purified and cDNA reverse transcribed as previously described (Barson et al., 2009, Chang et al., 2012). Resulting A_{260}/A_{280} ratios of total RNA from all animals ranged between 2.03 and 2.12. qRT-PCR was then performed as described (Barson et al., 2012a, Barson et al., 2012b). Levels of target gene expression were quantified relative to the level of cyclophilin, using the relative quantification method, and the average Ct

values for the endogenous control versus target genes differed by no more than 4 Ct. The primers, designed with ABI Primer Express V.1.5a software from published sequences, were: (1) cyclophilin: 5'-GTGTTCTTCGACATCACGGCT-3' (forward) and 5'-CTGTCTTTTGGAAGTTTGTCTGCA-3' (reverse); (2) MCH: 5'-ATCGGTTGTTGCTCCTTCTCTG-3' (forward) and 5'-TCTGCTTGGAGCCTGTGTTCTT-3' (reverse); (3) CCL2: 5'-GTGCTGTCTCAGCCAGATGCAGTT-3' (forward) and 5'-AGTTCTCCAGCCGACTCATTGGG-3' (reverse); and (2) CCR2: 5'-GGGGGCCACCACACCGTATGAC-3' (forward) and 5'-AGGGAGTAGAGTGGGGGCAGGAT-3' (reverse). Concentration of the MCH, CCL2 and CCR2 primers was 100 nM and of the CYC primer was 200 nM.

5-Bromo-2-deoxyuridine (BrdU) injection

To label proliferating cells in the embryonic hypothalamus, the control, 1 g/kg/day ethanol, and 3 g/kg/day ethanol dams were given intraperitoneal (i.p.) injections, every 8 h over 4 days, of BrdU (20 mg/kg, Sigma, St. Louis, MO) in 0.9% NaCl and 0.007 N NaOH, as described in our prior studies (Chang et al., 2008, Chang et al., 2012, Chang et al., 2013). These injections were given from E12–E15, the period of peak cell birth in the LH (Altman and Bayer, 1978, 1990). Comparison of the offspring of BrdU-injected (20 mg/kg) and non-injected dams has revealed no difference in the density of neurons in the hypothalamus or in the effects of prenatal ethanol on peptide expression, consistent with other reports showing little effect on development at higher doses of BrdU (Orikasa et al., 2010, Imamura and Greer, 2015). The offspring were sacrificed at P25, and their brains were processed as described previously (Chang et al., 2008, Chang et al., 2012, Chang et al., 2013).

Single-labeling immunofluorescence histochemistry (IF)

Immunofluorescence histochemistry was used, as previously described (Chang et al., 2008, Chang et al., 2013), to characterize the distribution pattern and to quantify the cell density of MCH, CCL2, and CCR2 in the LH of preadolescent rats, the area outlined in the photomicrograph in Fig. 1. Briefly, offspring at P25 ($n = 7/\text{group}$) were decapitated, and their brains were removed, immediately fixed in 4% paraformaldehyde at 4 °C for 48–72 h, and then cryo-protected in 25% sucrose at 4 °C for 60–72 h. Afterwards, brains were frozen and stored at -80 °C. Brains were cut at 30 μm with a cryostat, and free-floating coronal sections were processed with primary antibodies against MCH, CCL2 and CCR2 and then corresponding secondary antibodies (Table 1). Sections were viewed, and fluorescence images were captured using a Zeiss fluorescence microscope with MetaVue software. The density of immunofluorescent cells, in 8–12 images collected in each area in each animal, was quantified with Image-Pro Plus software (Version 4.5; Media Cybernetics) as described (Chang et al., 2008, Chang et al., 2012, Chang et al., 2013) and is reported as cells/ μm^2 .

Double-labeling and triple-labeling immunofluorescence histochemistry (IF)

Double-labeling IF was used to examine the co-existence of CCR2 with NeuN (marker of mature neurons) or MCH in the LH or of BrdU with CCR2, NeuN or MCH in the LH, to determine the proportion of CCR2 cells that are neurons or contain a specific orexigenic

peptide, MCH, or the proliferation of CCR2 or MCH neurons in the LH. Triple-labeling IF was used to further examine the proliferation of CCR2 neurons that co-label BrdU and MCH in the LH. Double- and triple-labeling IF was performed using different specific combinations of primary antibodies and their corresponding secondary antibodies based on our previous procedures (Chang et al., 2008, Chang et al., 2012, Chang et al., 2013). The combinations of primary antibodies and the corresponding combinations of secondary antibodies for double-labeling and triple-labeling IF are listed in Tables 2 and 3, respectively. For analysis of double- or triple-labeled CCR2⁺ cells with the other markers or MCH, the images were captured with a 20× objective, and the double- and triple-labeled cells were confirmed with a 40× objective and further validated by confocal Z-sectioning with a 40× water-immersion lens on a Zeiss LSM 510 META confocal microscope. The double-labeled cells were counted and reported as percentage of total single-labeled cells, and the triple-labeled CCR2/MCH/BrdU neurons were counted and reported as percentage of total CCR2/MCH neurons.

Data Analysis

Differences in the effects of prenatal ethanol on single-labeled MCH, CCL2 and CCR2 cells or on CCR2 cells that co-labeled NeuN, MCH and/or BrdU within each treatment group, were tested with a one-way ANOVA, followed-up by Tukey's post-hoc tests when appropriate. Differences in the effect of gestational ethanol on ethanol intake, ethanol preference, and ethanol reinstatement were determined with a two-way ANOVA, with repeated measures followed up by post-hoc comparison using Tukey's HSD. Data were determined to be distributed normally using the Shapiro-Wilk test. Significance was determined at $p < 0.05$, and data are reported as mean $\pm$ standard error of the mean (S.E.M.).

RESULTS

The overall goal of this study is to determine whether prenatal exposure to ethanol, at a relatively low dose that increases ethanol drinking in adolescent offspring, can stimulate neurogenesis in the embryo and increase in preadolescent rats the density of neurons that contain MCH, the chemokine CCL2, or its receptor CCR2, and also of neurons that co-localize MCH with CCL2 or CCR2.

Experiment 1: Effect of prenatal ethanol on consumption of and preference for ethanol in adolescent rats

This experiment tested the possibility that gestational exposure to relatively low levels of ethanol increases alcohol consummatory behavior during early adolescence (starting at P40), using a two-bottle choice paradigm under an intermittent access schedule. In the adolescent offspring of dams that received intraoral infusion of ethanol (1 or 3 g/kg/day) vs an isocaloric maltose-dextrin control solution ($n = 8/\text{group}$) from E10–E15, three measurements were taken of ethanol drinking, ethanol preference, and then ethanol intake following reinstatement after 14 days of abstinence. Analysis of their ethanol consummatory behaviors, collapsed across these three measurements, revealed an overall main effect of prenatal ethanol compared to control ($F(1,27) = 201.82, p < 0.01$), with no effects of ethanol on water intake ($F(1,27) = 0.04, ns$) or food intake ($F(1,27) = 0.04, ns$), and also a

significant interaction between the prenatal treatment and ethanol consummatory behaviors ($F(4,54) = 4.93, p < 0.05$). Specifically, prenatal exposure to ethanol had significant effects on the measures of ethanol intake ($F(2,29) = 9.22, p < 0.01$), ethanol preference ($F(2,29) = 7.28, p < 0.05$), and ethanol intake after reinstatement ($F(2,29) = 4.45, p < 0.01$) (Fig. 2). Post-hoc analyses showed that, compared to control, the ethanol-exposed offspring at both doses of gestational ethanol: 1) drank significantly more ethanol (+110%, $p < 0.05$); 2) exhibited an increased preference for the ethanol solution compared to water (+100%, $p < 0.05$); and 3) drank significantly more ethanol (+75%, $p < 0.05$) when it was reinstated after 14 days of abstinence. No differences were evident between the effects of these two ethanol doses on any of these measures (*ns*). Together, these findings demonstrate that a low level of ethanol exposure during gestation has clear, stimulatory effects on the ethanol-related consummatory behaviors of adolescent offspring.

Experiment 2: Effect of prenatal ethanol exposure on MCH peptide in LH of preadolescent rats

To determine whether prenatal ethanol exposure stimulates the neuropeptide, MCH, which is known to increase the consumption of ethanol (Morganstern et al., 2010b), this experiment examined preadolescent offspring (P25) of dams that received intraoral infusions of ethanol (1 or 3 g/kg/day) vs control isocaloric maltose-dextrin solution ($n = 7/\text{group}$) from E10–E15. Analyses of MCH in the LH of the preadolescent rats revealed an overall main effect of prenatal ethanol on MCH mRNA levels measured using qRT-PCR ($F(2,20) = 15.89, p < 0.05$) and on the density of MCH⁺ neurons measured using IF ($F(2,20) = 27.29, p < 0.05$). Post-hoc analyses showed that, compared to control, prenatal ethanol caused a significant increase in MCH mRNA at both doses of 1 g/kg/day (+70%, $p < 0.05$) and 3 g/kg/day (+98%, $p < 0.05$). There was a similar increase in the density of MCH⁺ neurons at 1 g/kg/day (+86%, $p < 0.05$) and 3 g/kg/day (+142%, $p < 0.05$) compared to control (7.47×10^{-5}) as illustrated in the photomicrographs (Fig. 3). Comparisons between the two doses (3 vs 1 g/kg/day) revealed significant differences between the groups, for both measures of mRNA levels (+18%, $p < 0.05$) and MCH⁺ neuronal density (+30%, $p < 0.05$). These data provide the first evidence that ethanol during pregnancy at low doses has a stimulatory effect on MCH neurons in the LH and demonstrate that this effect is long lasting, seen in preadolescent offspring six weeks after their exposure to ethanol and shortly before the behavioral change toward excess ethanol consumption in adolescent rats observed in Experiment 1.

Experiment 3: Effect of prenatal ethanol exposure on CCL2/CCR2 system in LH of preadolescent rats

With little known about the effect of gestational ethanol exposure on the CCL2/CCR2 chemokine system in the hypothalamus, this experiment measured in the LH levels of both mRNA or protein of CCL2 and CCR2 in preadolescent rats at P25 ($n = 7/\text{group}$) born from dams that received intraoral infusion of ethanol (1 or 3 g/kg/day) vs control isocaloric maltose-dextrin solution from E10–E15. Whereas CCL2⁺ cells have been described in the hypothalamus of adult rats (Banisadr et al., 2005; Poon et al., 2014), we observed in preadolescent rats relatively low levels of CCL2 mRNA and low density of lightly stained CCL2⁺ cells in control rats that showed little change (+1.6%) in response to gestational

ethanol at 1 g/kg/day ($F(2,20) = 0.03$, *ns*) or 3 g/kg/day ($F(2,20) = 0.624$, *ns*) compared to control. In contrast to CCL2, measurements of CCR2 revealed a dense population of CCR2⁺ cells in the LH and significant main effects of prenatal ethanol on CCR2 mRNA expression ($F(2,20) = 9.47$, $p < 0.01$) and on the density of CCR2⁺ cells ($F(2,20) = 12.86$, $p < 0.01$). Post-hoc analyses showed that, compared to control, ethanol induced a significant increase in levels of CCR2 mRNA, at both doses of 1 g/kg/day (+45%, $p < 0.05$) and 3 g/kg/day (+49%, $p < 0.05$). There was also a similar increase in the density of CCR2⁺ cells, at 1 g/kg/day (+66%, $p < 0.01$) and 3 g/kg/day (+74%, $p < 0.01$), as illustrated in the photomicrographs (Fig. 4), with no differences seen between the two ethanol groups (*ns*) for both measures. Examination of the cell type of the CCR2⁺ cells in the LH demonstrated that CCR2 colocalizes with NeuN, a marker of mature neurons (Fig. 5). This analysis revealed a main effect of ethanol on the density of mature CCR2 neurons, as indicated by the greater percentage of double-labeled CCR2⁺/NeuN⁺ cells relative to total CCR2⁺ cells ($F(2,20) = 44.63$, $p < 0.01$) (Fig. 5A). This stimulatory effect of ethanol, observed similarly at both doses ($p < 0.01$ vs control; *ns* for 3 vs 1 g/kg/day), was very strong as illustrated in the photomicrograph (Fig. 5B), with the percentage of CCR2⁺/NeuN⁺ neurons increased to as high as 87% ($p < 0.01$). Together this evidence demonstrates that CCR2 neurons in the LH, in contrast to CCL2 cells at this young age, are highly sensitive to ethanol during gestation which has a strong, stimulatory effect on their density in preadolescent rats.

Experiment 4: Effect of prenatal ethanol exposure on CCR2/MCH neurons in LH of preadolescent rats

Building on the strong effect of ethanol on CCR2 neurons in the LH, this experiment tested whether this chemokine receptor co-localizes with MCH and, if so, whether the density of these CCR2⁺/MCH⁺ neurons in preadolescent rats (P25) is affected by prenatal exposure to ethanol (1 or 3 g/kg/day) vs the control isocaloric maltose-dextrin solution ($n = 7$ /group) from E10–E15. Compared to control rats that exhibited only a few double-labeled neurons, there was a significant main effect of prenatal ethanol on the density of CCR2⁺/MCH⁺ cells relative to single-labeled CCR2⁺ cells ($F(2,20) = 35.82$, $p < 0.01$) and MCH⁺ neurons ($F(2,20) = 26.04$, $p < 0.01$) in the LH (Fig. 6A), as illustrated in the photomicrographs (Fig. 6B). Both doses of ethanol had a similar stimulatory effect on CCR2⁺/MCH⁺ neurons ($p < 0.01$ vs control; *ns* for 3 vs 1 g/kg/day), with almost all (83%) of the CCR2⁺ neurons found to contain MCH in these ethanol-treated rats. These results reveal a marked effect of prenatal ethanol on these double-labeled neurons in the LH of preadolescent rats, suggesting a close relationship between these chemokine and orexigenic peptide systems.

Experiment 5: Effect of prenatal ethanol on the genesis of CCR2, MCH and CCR2/MCH neurons in LH

This experiment investigated whether ethanol during gestation stimulates in the embryo the proliferation of MCH peptide neurons, as previously shown for OX in the LH (Chang et al., 2012), and also of CCR2 and CCR2/MCH double-labeled neurons. We examined the P25 offspring of dams that received intraoral infusion of ethanol (1 or 3 g/kg/day) or control isocaloric maltose-dextrin solution ($n = 5$ /group) from E10–E15 and also injections of the proliferation marker, BrdU, from E12–E15 as previously described (Chang et al., 2012, Chang et al., 2013). Whereas the control rats had very few CCR2⁺/BrdU⁺ or MCH⁺/BrdU⁺

cells, there was a significant main effect of ethanol on the genesis of CCR2⁺ ($F(2,20) = 25.07, p < 0.05$) and MCH⁺ ($F(2,20) = 14.17, p < 0.05$) cells, as indicated by a far greater percentage of double-labeled CCR2⁺/BrdU⁺ and MCH⁺/BrdU⁺ cells relative to single-labeled CCR2⁺ or MCH⁺ cells, respectively (Fig. 7). There was also a significant main effect of ethanol on the proliferation of CCR2⁺/MCH⁺ double-labeled neurons in the LH (Fig. 8), with triple-labeled CCR2⁺/MCH⁺/BrdU⁺ neurons absent in control rats and these newly generated neurons accounting for 35% of the population of CCR2⁺/MCH⁺ neurons ($F(2,14) = 36.03, p < 0.05$) and BrdU⁺ cells ($F(2,14) = 19.14, p < 0.05$), an effect observed similarly at both doses ($p < 0.01$ vs control; *ns* for 3 vs 1 g/kg/day). These findings in preadolescent rats demonstrate that prenatal ethanol increases the birth in the embryo of CCR2 and MCH neurons in the LH and also of neurons that contain both CCR2 and MCH, with few such proliferating neurons detected in control rats.

DISCUSSION

Clinical reports have shown gestational ethanol exposure to increase the consumption of alcohol and induce alcohol-related problems in young adults (Baer et al., 2003, Alati et al., 2006, Malone et al., 2010), and this phenomenon has also been demonstrated in rodents (Pueta et al., 2008, Youngentob and Glendinning, 2009, Diaz-Cenzano and Chotro, 2010, Fabio et al., 2013b, Cullere et al., 2014, Fabio et al., 2015). These animal studies suggest that the increase in ethanol intake is more likely to occur when ethanol is administered *in utero* at lower concentrations that increase BEC to levels <150 mg/dL and also when consumption is measured during early adolescence, at 30–50 days of age (Abel and York, 1979, Reyes et al., 1985, Chotro and Arias, 2003, March et al., 2009, Youngentob and Glendinning, 2009, Fabio et al., 2013a, Fabio et al., 2015) when there is increased risk for drug abuse (Caetano and Cunradi, 2002, McGorry et al., 2011). The present study supports this suggestion, showing this phenomenon to occur in young adolescent rats using a two-bottle choice paradigm under an intermittent access schedule. It provides new evidence that the excess ethanol drinking can occur when the embryo is exposed to ethanol at a dose as low as 1 g/kg/day, which leads to BEC of <100 mg/dL, and also during a brief period, from E10–E15, when neuronal development is greatest in the LH (Brischoux et al., 2001). These results are also notable in demonstrating that the greater ethanol drinking induced by gestational ethanol, averaging 7.5 g/kg/day that should induce pharmacologically relevant BEC (Simms et al., 2010, Barson et al., 2015), persists through adolescence and during reinstatement after 14 days of abstinence. Thus, just six days of exposure to blood alcohol levels of <100 mg/dL are sufficient to promote excess drinking behavior during adolescence.

With this behavioral disturbance found to be induced by our paradigm of prenatal ethanol administration, we next investigated whether it can be linked to changes in MCH peptide neurons in the hypothalamus. Consistent with our finding that prenatal exposure to ethanol stimulates the development of OX neurons in the LH (Chang et al., 2012), we provide new evidence here that ethanol during pregnancy for 6 days, at 1 or 3 g/kg/day, similarly stimulates MCH, both mRNA and neuronal density, in the LH of preadolescent rats. This stimulatory effect on MCH mRNA and neuronal density is not evident at higher ethanol doses (>6g/kg/day) (Chometton et al., 2014), possibly because these doses cause a reduction in cell density, cell proliferation and brain volume (Miller, 1995, Livy et al., 2003, Gil-

Mohapel et al., 2010) that interferes with the stimulatory effect shown here at low ethanol doses. Our results also demonstrate that gestational ethanol stimulates the proliferation of MCH neurons, as shown by an increased density of MCH⁺/BrdU⁺ double-labeled neurons in the offspring, indicating that these MCH neurons as they develop in the embryo are highly sensitive to low doses of ethanol, similar to the OX neurons in the LH (Chang et al., 2012). It is this increased genesis of MCH-expressing neurons *in utero* that leads to the greater expression and production of this peptide that persists long after the period of ethanol exposure, possibly with long-term behavioral consequences.

The evidence that MCH neurons colocalize with CCL2 (Banisadr et al., 2005) led us to test whether prenatal exposure to ethanol also affects the CCL2/CCR2 chemokine system in the LH. Evidence in adult rats shows that ethanol stimulates the CCL2/CCR2 system in the periphery and in different areas of the forebrain (He and Crews, 2008, Kane et al., 2013, Kane et al., 2014, Pascual et al., 2015), and there are a few reports showing prenatal or early postnatal ethanol administration to increase cytokine levels in the brain, such as transforming growth factor- β 2 in the cortex and hypothalamus of the offspring (Miller, 2003, Sarkar et al., 2007) and tumor necrosis factor- α and interleukin-6 in the whole embryo (Vink et al., 2005, Roberson et al., 2012). Little is known, however, about the effects of prenatal ethanol exposure on the CCL2/CCR2 signaling system in the brain, with only one study indicating that ethanol during pregnancy increases maternal levels of CCL2 in the circulation (Nanji et al., 2001). Thus, our results in preadolescent rats are the first to demonstrate that gestational ethanol has strong effects on this chemokine system, particularly CCR2, in the hypothalamus. Whereas the levels of CCL2 mRNA and density of CCL2⁺ cells in the LH are low at this young age and show little change in response to ethanol, baseline levels of CCR2 mRNA and density of CCR2⁺ cells are high and markedly increased by prenatal ethanol exposure. Notably, most of these CCR2⁺ cells in ethanol-treated rats are found to be mature neurons that co-label NeuN, showing them to be highly sensitive to ethanol in the same hypothalamic area where MCH neurons are significantly stimulated.

Our results further demonstrate a close relationship between CCR2 and MCH as they exist within the same neurons of the LH. In contrast to CCL2-labeled cells in preadolescent rats that are sparse and unaffected by ethanol, the CCR2 cells are found to be dense in the LH and to colocalize extensively with MCH, providing anatomical evidence closely relating these chemokine and neuropeptide systems. The effect of prenatal ethanol, even at 1 g/kg/day, on these CCR2 neurons that contain MCH is strong, with their density in preadolescent rats markedly increased to approximately 80% of the total CCR2 population in the LH and the birth of these CCR2/MCH neurons in the embryo, which are barely evident in control rats, also strongly stimulated by ethanol. These findings underscore the profound effects that ethanol, when provided during the few days critical for hypothalamic neurogenesis, can have on the development of these neuronal systems. The finding that gestational ethanol increases maternal levels of CCL2 in the circulation (Nanji et al., 2001) suggests the possibility that this chemokine has a role in stimulating the genesis of these CCR2 neurons that co-express MCH in the LH. In a recent report, we demonstrated that CCL2 *in vitro* stimulates the development and migration of hypothalamic neurons (Poon et al., 2014), and

there are other *in vitro* and *in vivo* studies showing that this chemokine can stimulate the proliferation of neuronal stem cells and neurons and the differentiation of neurons (Lawrence et al., 2006, Liu et al., 2007, Chintawar et al., 2009, Magge et al., 2009, Poon et al., 2014, Hong et al., 2015). This evidence introduces the possibility that the maternal chemokine system may be contributing to ethanol's stimulatory effects on the development of the CCR2/MCH co-labeled neurons in the LH.

Whereas the focus of this study is on neurons that co-label CCR2 and MCH, the possibility that non-neuronal cells are also involved needs to be considered. While the CCL2/CCR2 chemokine system is known to have effects on glia and microglia as well as neurons (El-Hage et al., 2006, McMillin et al., 2014, Poon et al., 2014), anatomical evidence in the brain is limited, showing CCR2 under normal conditions to exist on both neurons and astrocytes but not oligodendrocytes or microglia in the cortex (Banisadr et al., 2002) and only on neurons but not astrocytes or microglia in the hippocampus (Foresti et al., 2009). Further studies of the hypothalamus are needed, although our preliminary tests with the astrocyte marker, GFAP, or the microglia markers, Iba-1, ED-1 or OX-42, have so far failed to reveal any co-labeling with CCR2. Also, although detected in immune cells in the periphery (Lakaye et al., 2009), we find that MCH in the hypothalamus exists primarily in neurons and not astrocytes or microglia, and that these MCH-expressing neurons are highly responsive to prenatal ethanol exposure. Whereas we cannot fully eliminate the possibility that other cell types are involved, a predominant effect of prenatal ethanol at low doses on neurons is further supported by our published evidence, showing prenatal ethanol to have no effect on the density of astrocytes in the hypothalamus (Chang et al., 2012), and also by our findings here, showing a strong, stimulatory effect of prenatal ethanol on CCR2-positive cells that are almost exclusively neurons and that also co-label MCH.

This ethanol-induced increase in the genesis of CCR2 neurons that contain MCH, resulting in increased density of CCR2/MCH neurons in the LH of preadolescent rats, leads us to question whether this neuronal change has behavioral consequences, perhaps contributing to the greater ethanol drinking and stronger preference shown here during adolescence. Pharmacological studies demonstrate that central injection of MCH increases ethanol intake (Duncan et al., 2005, Morganstern et al., 2010b) while an MCH antagonist reduces it (Cippitelli et al., 2010), and endogenous expression of MCH is greater in rats prone to consuming excess ethanol (Morganstern et al., 2010b). Clinical studies also provide support for a role of MCH in mediating the rewarding properties of ethanol (DiLeone et al., 2003, Duncan et al., 2005, Kim et al., 2012). In addition, there is evidence that the CCL2/CCR2 signaling pathway itself has a role in controlling ethanol consumption, with studies showing a deletion of the CCR2 gene in males and CCL2 gene in females to reduce the drinking of ethanol (Blednov et al., 2005). Thus, together with this evidence, the present findings showing a marked increase in the density of CCR2 neurons that contain MCH neurons support the possibility that this chemokine system functions in close relation to this orexigenic peptide in promoting excess ethanol consumption during adolescence.

CONCLUSION

It is clear from these results that gestational exposure to ethanol, at a low dose of 1 g/kg/day for only 6 days, has a strong stimulatory effect in the embryo on the genesis of neurons that are programmed to express or coexpress CCR2 and the orexigenic peptide MCH in the LH and that these neuronal effects persist in the absence of ethanol and are evident in preadolescent rats. The high density in ethanol-treated rats of neurons that co-express CCR2 and MCH indicates that they are likely to have behavioral effects, including an increase in alcohol drinking behavior. Also, the high percentage of CCR2 neurons in the LH found to contain MCH supports a close and perhaps functional relationship between these chemokine and orexigenic peptide systems. The possibility that CCL2 interacts with and stimulates MCH through CCR2 is consistent with a recent study showing that CCL2 stimulates hypothalamic neurons and specifically those that express the orexigenic peptide, enkephalin (Poon et al., 2014). Further, the evidence that ethanol increases CCL2 levels in the dam (Nanji et al., 2001) suggests a role for maternal CCL2 in stimulating the development of CCR2 neurons in the LH, including the dense population that contain MCH.

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- Maternal drinking of low ethanol increases ethanol drinking in adolescent offspring
- Prenatal ethanol increases MCH in lateral hypothalamus of preadolescent offspring
- Prenatal ethanol increases CCR2 in lateral hypothalamus of preadolescent offspring
- Prenatal ethanol increases proliferation of CCR2 and MCH co-expressing neurons

Fig. 1.

Representative photomicrograph illustrating MCH labelled neurons in the LH area outlined with a dotted line that was examined in this study, shown at the anterior-posterior level of -3.36, from the Atlas of The Rat Brain, 5th Edition, by G. Paxinos and C. Watson, 2005. Abbreviations: LH, lateral hypothalamus; PeF, perifornical hypothalamus; F, fornix; V, ventricle; DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus.

Fig. 2.

Prenatal exposure to ethanol (1 or 3 g/kg/day) compared to control solution ($n = 10$ /group) increases ethanol intake and preference as well as ethanol intake after reinstatement in adolescent offspring. Data are mean $\pm$ S.E.M., * $p < 0.05$ vs control.

Fig. 3.

Prenatal ethanol (1 g/kg/day) compared to control solution ($n = 7/\text{group}$) increases the density of MCH^+ cells, as assessed by immunofluorescence histochemistry and illustrated here in the LH of preadolescent offspring (P25). For illustration of LH area, see Fig. 1. Scale bar = 100 μm .

Fig. 4. Prenatal ethanol (1 g/kg/day) compared to control solution ($n = 7/\text{group}$) increases the density of CCR2⁺ cells, as assessed by immunofluorescence histochemistry and illustrated here in the LH of preadolescent offspring (P25). For illustration of LH area, see Fig. 1. Scale bar = 100 μm

Fig. 5.

Prenatal ethanol (1 or 3 g/kg/day) compared to control solution ($n = 7/\text{group}$) increases the density of $\text{CCR2}^+/\text{NeuN}^+$ neurons, as assessed by immunofluorescence histochemistry and illustrated here in the LH of preadolescent offspring (P25). **A.** Prenatal ethanol increases the percentage of $\text{CCR2}^+/\text{NeuN}^+$ double-labeled neurons relative to total number of CCR2^+ single-labeled cells in the LH. **B:** Photomicrographs illustrate this effect of ethanol (1 g/kg/day) vs control in the LH, showing single-labeled CCR2^+ (red) and NeuN^+ (green) cells and double-labeled $\text{CCR2}^+/\text{NeuN}^+$ neurons (red CCR2 perikarya with green NeuN nucleus) identified by arrowheads. Images on the bottom are higher magnifications of cells located within the white square in the above image of an ethanol-treated rat. For illustration of LH area, see Fig. 1. Scale bar = 100 μm . Data are mean $\pm$ S.E.M., * $p < 0.05$ vs control.

Fig. 6.

Prenatal ethanol (1 or 3 g/kg/day) compared to control solution ($n = 7/\text{group}$) increases the density of $\text{CCR2}^+/\text{MCH}^+$ neurons, as assessed by immunofluorescence histochemistry and illustrated here in the LH of preadolescent offspring (P25). **A.** Prenatal ethanol increases the percentage of $\text{CCR2}^+/\text{MCH}^+$ double-labeled neurons relative to the total number of CCR2^+ (left) or MCH^+ (right) single-labeled cells. **B:** Photomicrographs illustrate this effect of ethanol (1 g/kg/day) vs control in the LH, showing single-labeled CCR2^+ (red) and MCH^+ (green) cells and double-labeled $\text{CCR2}^+/\text{MCH}^+$ (yellow) identified by arrowheads. Images on the far right are higher magnifications of a double-labeled neuron within the white square of an ethanol-treated rat. For illustration of LH area, see Fig. 1. Scale bar = 100 μm . Data are mean $\pm$ S.E.M., * $p < 0.05$ vs control.

Fig. 7.

Prenatal ethanol (1 or 3 g/kg/day) compared to control solution ($n = 5/\text{group}$) stimulates in the embryo the proliferation of CCR2⁺ and MCH⁺ cells, as shown using double-labeling immunofluorescence histochemistry. This is demonstrated by a significant increase in preadolescent offspring (P25) in the percentage of CCR2⁺/BrdU⁺ and MCH⁺/BrdU⁺ double-labeled cells in the LH relative to the total population of single-labeled CCR2⁺ (left) and MCH⁺ (right) cells, respectively. For illustration of LH area, see Fig. 1. Data are mean $\pm$ S.E.M., * $p < 0.05$ vs control.

Fig. 8.

Prenatal ethanol (1 and 3 g/kg/day) compared to control solution ($n = 5/\text{group}$) stimulates in the embryo the proliferation of CCR2⁺/MCH⁺ double-labeled neurons, as assessed by triple-labeling immunofluorescence histochemistry and shown in preadolescent offspring (P25).

A: Prenatal ethanol increases the percentage of CCR2⁺/MCH⁺/BrdU⁺ triple-labeled neurons relative to the total number of CCR2⁺/MCH⁺ double-labeled neurons. Data are mean $\pm$ S.E.M., * $p < 0.05$ vs control. **B:** Photomicrographs illustrate this effect of ethanol (1 g/kg/day) vs control group in the LH, with triple-labeled CCR2⁺/MCH⁺/BrdU⁺ neurons (mixed green/aqua/yellow) identified by arrowheads. Images on the bottom right are higher magnifications of single-labeled CCR2⁺ (red), MCH⁺ (green), and BrdU⁺ (blue) cells and triple-labeled CCR2⁺/MCH⁺/BrdU⁺ neurons located within the white square of an ethanol-treated rat. For illustration of LH area, see Fig. 1. Scale bar = 100 μm . Data are mean $\pm$ S.E.M., * $p < 0.05$ vs control.

Table 1

Antibodies used in single immunofluorescence histochemistry. Vendor for secondary antibodies:
JacksonImmunoResearch Laboratories, Inc, PA

Primary antibody	Working concentration	Vendor	Secondary antibody	Working concentration
Rat anti-BrdU, monoclonal	1/200	Abcam, MA	Cy5-Donkey anti-Rat IgG	1/100
Rabbit anti-MCH	1/200	Santa Cruz, CA	FITC-Donkey anti-Rabbit IgG	1/50
Goat anti-CCR2	1/100	Santa Cruz, CA	Cy3-Donkey anti-Goat IgG	1/100
Goat anti-CCL2	1/100	Santa Cruz, CA	Cy3-Donkey anti-Goat IgG	1/100

Table 2

Antibodies used in double-immunofluorescence histochemistry. Vendor for secondary antibodies: JacksonImmunoResearch Laboratories, Inc, PA

Combination	Primary antibody	Working concentration	Vendor	Secondary antibody	Working concentration
CCR2+BrdU	Goat anti-CCR2	1/100	Santa Cruz, CA	Cy3-Donkey anti-Goat IgG	1/100
	Rat anti-BrdU	1/100	Abcam, MA	Cy5-Donkey anti-Rat IgG	1/100
MCH+BrdU	Rabbit anti-MCH	1/200	Santa Cruz, CA	FITC-Donkey anti-Rabbit IgG	1/50
	Rat anti-BrdU	1/100	Abcam, MA	Cy5-Donkey anti-Rat IgG	1/100
NeuN+BrdU	Mouse anti-NeuN,	1/50	Millipore-Chemicon, CA	FITC-Donkey anti-Mouse IgG	1/50
	Rat anti-BrdU monolonal	1/100	Abcam, MA	Cy5-Donkey anti-Rat IgG	1/50
CCR2+MCH	Goat anti-CCR2	1/100	Santa Cruz, CA	Cy3-Donkey anti-Goat IgG	1/100
	Rabbit anti-MCH	1/200	Santa Cruz, CA	FITC-Donkey anti-Rabbit IgG	1/50
CCR2+NeuN	Goat anti-CCR2	1/100	Santa Cruz, CA	Cy3-Donkey anti-Goat IgG	1/100
	Mouse anti-NeuN, monolonal	1/50	Millipore-Chemicon, CA	FITC-Donkey anti-Mouse IgG	1/50

Table 3

Antibodies used in triple-immunofluorescence histochemistry. Vendor for secondary antibodies: JacksonImmunoResearch Laboratories, Inc, PA

Combination	Primary antibody	Working concentration	Vendor	Secondary antibody	Working concentration
CCR2+MCH+BrdU	Goat anti-CCR2	1/100	Santa Cruz, CA	Cy3-Donkey anti-Goat IgG	1/100
	Rabbit anti-MCH	1/200	Santa Cruz, CA	FITC-Donkey anti-Rabbit IgG	1/50
	Rat anti-BrdU	1/100	Abcam, MA	Cy5-Donkey anti-Rat IgG	1/100