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Ethanol Exposure during the Early First Trimester Equivalent Impairs Reflexive Motor Activity and Heightens Fearfulness in an Avian Model

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Abstract

Prenatal alcohol exposure is a leading cause of childhood neurodevelopmental disability. The adverse behavioral effects of alcohol exposure during the second and third trimester are well documented; less clear is whether early first trimester-equivalent exposures also alter behavior. We investigated this question using an established chick model of alcohol exposure. *In ovo* embryos experienced a single, acute ethanol exposure that spanned gastrulation through neuroectoderm induction and early brain patterning (19–22hr incubation). At 7 days post-hatch the chicks were evaluated for reflexive motor function (wingflap extension, righting reflex), fearfulness (tonic immobility), and fear/social reinstatement (open field behavior). Chicks exposed to a peak ethanol level of 0.23–0.28% were compared against untreated and saline-treated controls. Birds receiving early ethanol exposure had a normal righting reflex and a significantly reduced wingflap extension in response to a sudden descent. The ethanol-treated chicks also displayed heightened fearfulness, reflected in increased frequency of tonic immobility, and they required significantly fewer trials for its induction. In an open field test, ethanol treatment did not affect latency to move, steps taken, vocalizations, defecations, or escape attempts. The current findings demonstrate that early ethanol exposure can increase fearfulness and impair aspects of motor function. Importantly, the observed dysfunctions resulted from an acute ethanol exposure during the period when the major brain components are induced and patterned. The equivalent period in human development is 3–4 weeks post-conception. The current findings emphasize that ethanol exposure during the early first trimester equivalent can produce neurodevelopmental disability in the offspring.

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Keywords

fetal alcohol spectrum disorders; chick embryo; tonic immobility; motor coordination; fearfulness; neurobehavior

Introduction

Prenatal alcohol exposure (PAE) can cause Fetal Alcohol Spectrum Disorders (FASD), a major cause of neurodevelopmental disability that affects 2–5% of live births and costs ~\$3.6 billion annually (Centers for Disease Control and Prevention, 2009; Lupton et al., 2004; May et al., 2009). FASD can include somatic growth deficits and distinctive craniofacial and other physical anomalies. However, the major challenges confronting affected individuals are lifelong impairments in learning, attention, impulse control, memory, and motor skills (Jones et al., 1973; Streissguth et al., 2000). Alcohol exposure during all three trimesters causes significant behavioral and learning deficits, and these outcomes are improved if alcohol consumption is stopped or reduced during the first or second trimester (Autti-Rämö et al., 1992; Coles et al. 1985, 1987). It is less clear whether PAE causes behavioral deficits following binge exposure during the first month of pregnancy, a period when the condition often is unrecognized. Ethanol exposure during the early first trimester period causes craniofacial, cardiac, and brainstem deficits (Debelak and Smith, 2000; Dunty et al. 2001; Maier et al. 1997). The potential of PAE to adversely affect neurobehavior during the early developmental period is an important question because 10.8%–13.7% of non-pregnant women aged 18–44 yrs report binge-drinking behavior (Denny et al. 2009).

We addressed this question in a well characterized chick embryo model that displays craniofacial deficits similar to those of FASD (Debelak and Smith, 2000). Embryos receive an acute ethanol challenge at the onset of gastrulation (19 hr incubation) via yolk injection. Embryonic ethanol levels peak 0.5–2 hr later at 50–60 mM (0.23–0.28%) and then rapidly dissipate as the ethanol equilibrates between embryo, white and yolk to a final level of ~9 mM (0.04%). During the next week ~50% of the ethanol leaves the egg via gas diffusion through the shell (Pennington et al., 1983). Detectable alcohol dehydrogenase activity is first detected at day 9 of incubation and rapidly removes the remaining ethanol (Pennington et al., 1983). Thus the model is largely one of acute binge ethanol exposure, but may also have influences from low-level chronic exposure during the organogenesis period.

The ethanol exposure protocol described here causes significant apoptosis within cranial neural crest populations and within progenitor neurons that reside in the presumptive forebrain, midbrain and hindbrain (Debelak and Smith 2000; Su et al., 2001). To test whether the early exposure model also caused neurobehavioral deficits, we challenged *in ovo* embryos with a single ethanol dose at gastrulation and evaluated multiple behaviors post-hatch. We report here that a single, acute ethanol exposure at gastrulation caused significant and selective motor impairments and enhanced the fearfulness of the hatched offspring.

Methods

Animals

Fertile white leghorn eggs were incubated at 37.5°C to gastrulation stage (19 hr incubation, stage 4; Hamburger and Hamilton, 1951). Eggs were injected into the yolk core through the blunt end (avoiding the embryo) with saline or 0.43 mmol ethanol in isotonic saline in a final volume of 250 µl; some eggs were uninjected and served as untreated controls. Eggs were sealed, reincubated and hatched using standard husbandry. Hatched chicks were

housed in a communal heated brooder with free access to food and water. Protocols were approved by the UW-Madison Research Animal Care Committee and experiments conducted according to the NIH Guide for the Care and Use of Laboratory Animals. Separate sets of chicks were hatched for measures of body weight and for the pilot open field test.

Behavioral Testing

Behavioral testing was performed on a single day on randomly selected individual birds in this order, open field, righting reflex/tonic immobility, and wingflap response, to minimize the effects of handling (Montevecchi et al., 1973). We chose tests that are well-validated in domestic poultry. Note that, because the chicken is a domesticated animal, the motivations (e.g. fearfulness, anxiety, social reinstatement) underlying these behaviors can differ from those of laboratory rodents (Forkman et al. 2007).

Novel Open Field Testing

At day 7 post-hatch, randomly selected chicks were individually transferred in a closed heated container to a quiet room. After acclimating for 30 min, the chick was placed in the center of a 60 × 60 cm novel arena with 30 cm high white sides and padded floor cover (Gallup and Suarez, 1980; Eddy and Gallup, 1990). Behavior was monitored for 5 min by treatment-blinded experimenters seated quietly approximately 0.5 m distant; observer positions and tasks did not vary during the study. The following behaviors were recorded: number of soft (peep) and loud (distress cry) vocalizations, latency to first step, number of steps, hops and stumbles, number of escape attempts, latency to defecation, and number of defecations. The arena was cleaned between trials.

Righting Reflex and Tonic Immobility

At the conclusion of the open field testing, the bird was placed supine on a padded table top under gentle restraint. The bird was released when its head relaxed; this seldom took longer than 3 sec. The time taken for the bird to right itself and stand after restraint removal was recorded; only those trials when the bird tried to right itself were used to calculate the median righting reflex time for the bird and trials that involved TI were excluded from that calculation. If a bird made no attempt to right itself after 10 sec, the bird was stood on its feet. A trial in which the bird made no attempt to right itself was considered an instance of tonic immobility, or the temporary loss of the righting reflex and severe motor inhibition (Gallup et al., 1971). Each bird was tested in a single session of 10 trials. The median righting reflex time and the number of instances of tonic immobility during the session were recorded.

Wingflap Response

After a 2 min rest period, the chick's balance and reflexes were tested using a standard protocol (Rager and Gallup, 1986). The chick was perched on the index finger of an extended hand; the experimenter's fingers securely anchored the feet so the bird did not fall. The hand was quickly and evenly lowered ~3 ft in 2–3 seconds (overhead to waist), stimulating chicks to extend wings and maintain balance. The degree of wing extension during the descent was visually scored by two observers using the criterion: 2 – wings fully extended, energetic flapping; 1.5 – wings extended but not quite fully or one wing extended and one not; 1 – partial wing extension; 0 – no wing extension. The trial was repeated 10 times with a 5 sec interval between trials, and the median response for each trial and the overall trial was calculated.

Statistics

Data are presented as either mean $\pm$ standard error or median $\pm$ range as indicated. Data were first evaluated using Shapiro-Wilk Normality Test (SigmaStat, Jandal Scientific Software). Data not normally distributed were analyzed as indicated using either the Mann-Whitney U Statistic or Kruskal-Wallis one-way analysis of variance by ranks followed-up with the non-parametric Dunn test on rank sums. Frequency data were analyzed using either Chi square or Fisher Exact test as indicated. Correlations were measured by Pearson product moment correlation. Data were not analyzed by sex because sex could not be determined at this young age. $P < 0.05$ was the criterion for significance.

Results

Hatch Parameters

Both saline and ethanol treatment equally reduced chick survival to hatch as compared with untreated eggs ($p = 0.027$, Table 1). This outcome was expected as any eggshell breach, even after sealing, can alter the egg's internal physiology and impair hatch (Romanoff, 1972). Ethanol did not affect the chick's ability to pip or hatch and only one saline and one ethanol chick required assistance to emerge from the shell. Ethanol treatment did not affect body size at hatch or growth rate during the 9 day post-hatch period as compared with untreated or saline-treated controls (Table 1). Injection itself did not impair growth.

Wingflap Response

We evaluated reflexive motor activation and coordination in response to a nonaversive stimulus. In the test, the bird experiences a rapid and controlled descent during which the instinct is to extend the wings and stabilize the body (Rager and Gallup, 1986). Kruskal-Wallis one way analysis of variance on ranks revealed a significant treatment effect ($H=15.64$ with two degrees of freedom, $p<0.001$). Saline-challenged chicks did not differ from untreated controls in their wingflap response (Figure 1; $Q=0.070$, $p>0.05$). In contrast, birds challenged with ethanol had a significantly reduced wingflap response and displayed on average more modest wing extensions as compared with saline-treated ($Q=3.366$, $p<0.01$) and untreated birds ($Q=3.471$, $p<0.01$), analyzed using the non-parametric Dunn test on rank sums. This suggested that reflexive motor activation and coordination might be adversely affected by a single ethanol challenge in early development.

Righting Reflex

To further probe motor reflexes and coordination, we tested the righting reflex of the chicks. Birds were placed on their back under gentle restraint and the time required to return to standing position was recorded. The median time to stand did not significantly differ between untreated, saline-treated and ethanol-treated chicks (Figure 2, $p>0.64$), indicating that ethanol did not impair this particular task.

Tonic Immobility

The righting reflex task also evaluated the birds' susceptibility to tonic immobility. Tonic immobility (TI) occurs when an animal is placed on its back and makes no attempt to right itself once the restraint is removed. In the domesticated chick TI is a well-validated response to fear; the accepted interpretation is that the animal feigns death in an attempt to evade a predator (experimenter) response, and both the duration of TI and the susceptibility to TI are positive measures of fear level (Gallup et al., 1971; reviewed in Forkman et al., 2007). During the righting reflex test, some birds would transition into TI. We found that untreated and saline-treated birds did not differ in the percentage of birds that transitioned at least once into TI (Figure 3A; $p=0.66$) and did not differ in the percentage of attempts that resulted in

TI (mean frequency of TI) (Figure 3B; $p=0.51$). The mean frequency of TI was $3.1\% \pm 6.0\%$ for untreated birds and $1.5\% \pm 3.8\%$ for saline-injected birds. In contrast, ethanol-treated birds were significantly more likely to display one or more instances of TI compared with saline-treated birds (Figure 3A; $p=0.046$), and during the ten trials they transitioned more frequently into TI ($15.7\% \pm 19.5\%$) as compared to saline-treated and untreated birds ($p < 0.001$; Figure 3B). Ethanol-treated birds also required significantly fewer trials to achieve the TI state (6.67 ± 3.48 trials; Figure 3C), as compared with untreated (10.19 ± 1.64 trials; $p=0.042$) and saline-treated birds (10.25 ± 1.39 trials, $p=0.017$). We did not measure the duration of TI as birds were aroused after 10 seconds of failing to right themselves.

Novel Open Field Behavior

The behavior of domestic poultry in the novel open field test reflects the influence of two motivations, fearfulness versus the need for social reinstatement (reviewed in Forkman et al., 2007; also see Gallup and Suarez 1980; Suarez and Gallup 1983; Jones and Carmichael 1997; Montevecchi et al., 1973). Behaviors such as long latency before first step, few steps taken, and few loud distress cries are strongly correlated with each other and have been interpreted to reflect heightened fear. A second set of behaviors including short latency before first step, many steps taken, and many loud vocalizations are similarly correlated and are generally believed to reflect the bird's desire to reunite with the flock, a behavior common in domesticated animals with high social needs. We first evaluated these behaviors in a distinct set of untreated chicks. The analysis revealed strong and negative correlations between latency to first step and the total number of steps taken ($R = -0.6558$; $p = 0.000924$), the total number of loud vocalizations ($R = -0.6612$, $p = 0.00081$), and the total number of escape attempts ($R = -0.63314$, $p = 0.00156$). The negative correlations between long latency to move versus increased movement/distress cries were consistent with the interpretation that these reflected different motivation states. That these behaviors segregated in a manner consistent with the established literature endorsed the test's validity.

We then repeated the novel open field test using our untreated, saline-treated and ethanol-treated chicks. We found that the mean and median values for the tested behaviors varied widely within the two control groups and within the ethanol-treated chicks, and this wide variance obscured potential behavioral differences between the three groups. Kruskal-Wallis one-way analysis of variance on ranks found no effect of ethanol treatment on the individual measures of latency to first step, total steps, and total loud vocalizations. Linear and non-linear regression analysis did not reveal modifying factors between the treatment groups. Ethanol treatment did not affect the strong, negative correlations between latency to first step versus total steps and loud vocalizations (data not shown). However, we observed that the ethanol-treated chicks had strongly dichotomized behavior and exhibited either long latency to move (defined as ≥ 240 sec, 7/14) with few distress vocalizations (defined as < 20 distress vocalizations, 7/14), or they exhibited short latency (< 60 seconds, 7/14) and many distress vocalizations (> 250 distress vocalizations, 7/14). In contrast, the chicks in both control groups exhibited a range of behaviors and were not dichotomized. The study lacked sufficient power to indicate whether this difference was significant. We conclude that this early model of ethanol exposure did not affect chick behavior in a novel open field.

Discussion

We present here the first report of enhanced fearfulness and impaired reflexive motor function in animals exposed to a single, acute ethanol dose during the period of gastrulation and early neurulation. Few studies have examined the behavioral consequences of ethanol exposure during this period, which is surprising because such exposures can cause pronounced brain dysmorphologies and neuroprogenitor losses that might be predictive of later dysfunctions (Debelak and Smith, 2000; Dunty et al. 2001; Maier et al., 1997; Yelin et

al., 2007). In humans, first trimester ethanol consumption produces milder cognitive deficits as compared with exposure in all trimesters; however, even those early exposures are associated with speech, math, and reading deficits, motor and reflexive anomalies, and increased activity, as compared with case controls (Autti-Ramo and Granstrom, 1991; Coles et al., 1985, 1991; Larsson et al., 1985). Our findings endorse the clinical evidence that first trimester PAE can have adverse neurodevelopmental consequences and support the position that there is no safe pregnancy period for alcohol consumption.

Our work further validates the avian model as an investigative tool for behavioral studies of developmental toxicant exposure and for ethanol specifically. Previous avian work on ethanol's behavioral effects emphasized learning outcomes and found that ethanol exposure during the early organogenesis period (days 0–4 incubation) impaired the acquisition of a detour learning task (Means et al., 1988). Ethanol also prevented long-term memory consolidation in a one-trial passive avoidance paradigm (Rao and Chaudhuri, 2007). The learning impairment was selective as short-term memory (Rao and Chaudhuri, 2007) and acquisition/extinction tasks (Dose et al., 1995) were unaffected. Here we expand that work to identify alterations in fearfulness and reflexive motor responses, and further endorse the avian model's utility for investigations of developmental ethanol exposure.

This study is the first to evaluate TI responses following developmental ethanol exposure. The increased susceptibility of ethanol-treated chicks to TI suggests the birds have increased fearfulness. TI in chick is hypothesized to be a protective response to extreme fear; studies find that a predator loses interest in the immobilized animal and this provides an escape opportunity (Forkman et al. and references therein). Heightened fearfulness is observed in rodent FASD models, with hypersensitivity to stress and slower habituation to stressors (Cohen et al., 1985; Osburn et al., 1998; Weinberg et al., 1996). The increased fearfulness is reflected in hypothalamic-pituitary-adrenal (HPA) hyperresponsivity, and the ethanol-exposed offspring have increased levels of corticosterone, adrenocorticotropin and β -endorphin in response to stressors (Osburn et al., 1998; Weinberg et al., 1996, 2008). Elevated fearfulness is observed in a primate FASD model (Schneider et al., 2004), and hyperreactivity to fear has been noted in individuals with FASD (Harris et al., 1993). Our findings regarding TI are in line with these other ethanol studies and endorse that early developmental ethanol exposure causes long-term changes in fear hyperreactivity and/or response inhibition.

TI engages the cerebral cortex, amygdala and brainstem and occurs in a wide range of vertebrates and possibly humans (Moskowitz, 2004). In avians, TI responses are mediated by the acropallium and pallial amygdala (formerly called the archistriatum), a region with homology to the mammalian amygdala (Davies et al., 1997; Masur et al., 1973; Saint-Dizier et al., 2009). Ethanol exposure during neural induction might not be expected to affect the brain regions that affect TI. However, the acropallium and pallial amygdala originate from the caudal telencephalon, which in turn arises from the prosencephalon or forebrain. In the avian embryo, the prosencephalon is the first brain region to be morphologically distinguished and emerges by the 9 somite stage (29–30 hrs incubation) at the ventral base of the anterior cerebral suture (Lillie, 1952), not long after ethanol challenge (19 hr). The ethanol exposure period encompasses events critical for prosencephalon formation, including head process emergence (which induces the anterior neuroectoderm), and the subsequent establishment of segmental identities within that neuroectoderm. These events can be perturbed by acute ethanol exposure (Yelin et al., 2007). Our results endorse prior observations in chick that single binge ethanol exposures early in development can adversely affect neurobehavioral outcomes.

This is also the first report to evaluate reflexive motor activities in ethanol-exposed chicks. Although their righting reflex times were normal, the ethanol-treated birds had impaired postural balance and attenuated wing extension in response to instability. Deficits in balance and coordination are also observed in children with prenatal alcohol exposure (Connor et al. 2006; Harris et al. 1993; Jirikowic et al. 2008; Roebuck et al. 1998). Whether this impairment is due to cerebellar dysfunction or to damage in another brain or spinal region was not determined. We note that the hindbrain including the presumptive cerebellum is induced and patterned during the ethanol exposure period and would be vulnerable to ethanol-induced damage. Indeed we and others observe ethanol-induced apoptosis within hindbrain neuronal precursors within this time period (Debelak and Smith, 2000; Dunty et al., 2001), and such losses could contribute to the deficits seen here.

Given that the ethanol-exposed chicks were more susceptible to TI induction, the absence of abnormal behavior in the novel open field is perhaps surprising. However, fear-related responses are complex and can be modified by factors such as environment and context. Domestic poultry are gregarious, diurnal, and prefer open over confined housing. Thus their fear responses to a novel environment (e.g. latency, steps) might have been modified by their need to rejoin the flock (vocalization, escape attempts). These birds were group-housed prior to testing, and this may have caused social reinstatement needs to dominate over the fearfulness caused by the novel open field. That we could recapitulate in our controls the same behavioral correlations in the open field that are reported in the literature (Forkman et al., 2007; Gallup and Suarez, 1980; Montevecchi et al., 1973) suggests that our testing protocol was valid. Interestingly, Means et al. (1988) reported an identical wide variance in the open field behavior of ethanol-exposed chicks but did not further analyze the data. While it is possible that ethanol effects might be found if the sample size was increased, overall these data indicate that early developmental ethanol exposure did not affect novel open field behavior in chick.

In summary, we report that developmental ethanol exposure during gastrulation and early neurulation led to increased fearfulness and impaired aspects of reflexive motor function. Importantly, these dysfunctions resulted from a single acute ethanol exposure during the early developmental period when the major brain components are induced and patterned. In humans this corresponds to weeks 3–4 post-conception, a time when the pregnancy may not be recognized and drinking behaviors are not yet curtailed. There may be additional contributions from the low, chronic ethanol exposure experienced during the subsequent week of organogenesis. Our findings emphasize that ethanol exposure during the early first trimester equivalent can produce neurobehavioral disability in the offspring.

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Figure 1.

Wingflap extension in response to sudden descent. Ethanol-treated chicks (E; N=14) had a significantly reduced response (*) as compared with untreated (N=16; $Q=3.471$, $p<0.01$) and saline-treated controls (N=13; $Q=3.366$, $p<0.01$) as determined by Kruskal-Wallis one-way analysis of variance on ranks with two degrees of freedom ($H=15.64$, $p<0.001$) followed by the non-parametric Dunn test on rank sums. Shown is the median response and range where 2=strong response, 1= weak response; 0 = absent response.

Figure 2.

Time (in seconds) required for chicks to right themselves after being placed on the back. Trials in which birds transitioned to TI were excluded. Ethanol-treated chicks (N=14) did not differ in the time needed to stand as compared with untreated (N=16) and saline-treated controls (N=13) as determined by Kruskal-Wallis one-way analysis of analysis of ranks with two degrees of freedom ($H=0.900$, $p=0.637$). Shown is the median response and range..

Figure 3.

Tonic immobility (TI) responses. **(A)** Significantly more ethanol-treated chicks (N=14) displayed at least one instance of TI as compared with saline-treated (N=13; $p=0.046$) but not untreated birds (N=16; $p = 0.135$) as determined by Fisher's exact test. **(B)** The mean frequency of TI per 10 trial session was significantly greater in ethanol-treated chicks as compared with saline-treated and untreated birds as determined by Chi-square analysis with two degrees of freedom ($\chi^2 = 26.845$, $p < 0.001$). **(C)** Significantly fewer trials were required to induce TI in ethanol-exposed chicks versus both untreated ($T = 252.5$; $p=0.042$) and saline-treated controls ($T = 225.0$; $p=0.017$), as determined by Mann-Whitney rank-sum test. Values in B and C are mean $\pm$ SD. * significantly different as defined in legend.

Table 1

Physical Parameters

Treatment	Hatch success%	Body weight at hatch, g	Body weight at day 9, g	% change in body weight
Untreated	70.0 (14/20)	38.08 $\pm$ 2.16	64.48 $\pm$ 5.20	59.90 $\pm$ 6.08
Saline	46.2 (24/52) *	39.59 $\pm$ 2.60	65.41 $\pm$ 6.13	60.95 $\pm$ 6.20
Ethanol	39.6 (19/48) *	39.37 $\pm$ 2.04	64.03 $\pm$ 4.94	61.90 $\pm$ 6.61

Values are means $\pm$ SD, with N indicated in parentheses

* Significantly differed from untreated control at $p = 0.027$ by Chi-square analysis with one degree of freedom. These chicks were not studied further.