Ethanol itself is a holoprosencephaly-inducing teratogen

Mingi Hong*, Robert S. Krauss

Department of Cell, Developmental, and Regenerative Biology, Icahn School of Medicine at Mount Sinai, New York, NY, United States of America

* Mingi.Hong@mssm.edu

Abstract

Ethanol is a teratogen, inducing a variety of structural defects in developing humans and animals that are exposed in utero. Mechanisms of ethanol teratogenicity in specific defects are not well understood. Oxidative metabolism of ethanol by alcohol dehydrogenase or cytochrome P450 2E1 has been implicated in some of ethanol’s teratogenic effects, either via production of acetaldehyde or competitive inhibition of retinoic acid synthesis. Generalized oxidative stress in response to ethanol may also play a role in its teratogenicity. Among the developmental defects that ethanol has been implicated in is holoprosencephaly, a failure to define the midline of the forebrain and midface that is associated with a deficiency in Sonic hedgehog pathway function. Etiologically, holoprosencephaly is thought to arise from a complex combination of genetic and environmental factors. We have developed a gene-environment interaction model of holoprosencephaly in mice, in which mutation of the Sonic hedgehog coreceptor, Cdon, synergizes with transient in utero exposure to ethanol. This system was used to address whether oxidative metabolism is required for ethanol’s teratogenic activity in holoprosencephaly. We report here that t-butyl alcohol, which is neither a substrate nor an inhibitor of alcohol dehydrogenases or Cyp2E1, is a potent inducer of holoprosencephaly in Cdon mutant mice. Additionally, antioxidant treatment did not prevent ethanol- or t-butyl alcohol-induced HPE in these mice. These findings are consistent with the conclusion that ethanol itself, rather than a consequence of its metabolism, is a holoprosencephaly-inducing teratogen.

Introduction

Holoprosencephaly (HPE) is a common congenital disorder in which the midline of the forebrain and/or midface is lacking [1]. HPE occurs with a frequency of about 1 in 250 conceptions, with ~97% of holoprosencephalic fetuses succumbing in utero [2] [3]. Presentation of HPE is extremely variable, with a spectrum of phenotypes ranging from failure to partition the forebrain into hemispheres to deficits in the midfacial midline [4, 5]. The most severe cases are not compatible with survival. However, cases with mild forebrain involvement are associated with mental deficiency [6, 7].

Both genetic and environmental factors are implicated in the etiology of HPE [5, 6, 8–10]. Heterozygous, loss-of-function mutations in the Sonic hedgehog (Shh) signaling pathway are
associated with HPE [10]. However, some mutation carriers have no obvious clinical manifestation, even in affected pedigrees [10–12]. In contrast, offspring who inherit such mutations are at high risk of HPE. Modeling of these observations has led to a multifactorial, “mutation plus modifier” paradigm, in which the phenotypic outcome associated with a heterozygous mutation is influenced by more common genetic variants and/or environmental exposures [13]. Among the non-genetic risk factors implicated in HPE is fetal alcohol exposure [8, 9]. Exposure to specific teratogens may be sufficient to cause HPE in some cases [14, 15]. However, it is likely that many structural birth defects are caused by a complex combination of genetic and environmental factors, which interact to disrupt morphogenetic events during development [16, 17].

We have modeled this type of phenomenon in mice. Cdon is coreceptor for Shh, binding directly to Shh and to other components of the Shh receptor, including the primary receptor, PTCH1, and the additional coreceptors, Boc and Gas1 [18–20]. Heterozygous, loss-of-function mutations in CDON have been identified in some HPE patients [18]. Cdon mutant mice develop HPE in a strain-dependent manner [21, 22]. Cdon mutants on a 129S6 background display only mild, mid-facial features of HPE with low penetrance. These mice have a sub-threshold defect of Shh signaling and are sensitized to HPE-modifying factors, including dosage-dependent loss of one of the other Shh coreceptor-encoding genes (Gas1 or Boc) [23, 24]. Significantly, 129S6 Cdon +/- mice developed a wide spectrum of HPE phenotypes, at high penetrance, upon in utero exposure to ethanol (EtOH); in contrast, wild type and heterozygous littermates did not display HPE [25].

Mechanisms of EtOH teratogenicity are not well understood. EtOH is oxidized to acetaldehyde by alcohol dehydrogenase (ADH), and acetaldehyde is oxidized to acetate by aldehyde dehydrogenase (ALDH). Acetate enters the carbon pool with some excreted as CO2 [26]. Many of EtOH’s toxic effects involve its metabolism by ADH and/or cytochrome P450 2E1 (Cyp2E1, which also produces acetaldehyde), with ADH responsible for the great majority of EtOH breakdown [26, 27]. Several potential mechanisms of ethanol teratogenesis require its oxidative metabolism. First, EtOH-derived acetaldehyde has been implicated in EtOH-induced exencephaly [28]. A second possible mechanism involves interference with retinoic acid (RA) synthesis. Similar to EtOH metabolism, vitamin A (retinol) is converted to RA by a two-step ADH/ALDH mechanism. EtOH and acetaldehyde may act as competitive inhibitors of the ADH/ALDH enzymes involved in RA production, with a resulting failure to produce sufficient levels of RA for normal developmental patterning [29–31]. Finally, while Cyp2E1 is quantitatively less important than ADH, EtOH metabolism by this enzyme produces both acetaldehyde and reactive oxygen species (ROS) [26, 27]. Generalized oxidative stress to exposed fetuses may therefore also contribute to EtOH-induced HPE. Mechanisms that do not require EtOH metabolism also exist. EtOH itself perturbs cellular membranes, and it can also bind and inhibit the function of specific membrane proteins, such as the cell adhesion molecule, L1 [32–36].

To begin to assess mechanisms of fetal alcohol-induced HPE, we tested the ability of t-butyl alcohol (Fig 1; IUPAC name, 2-Methylpropan-2-ol; abbreviated here as t-BuOH) to induce HPE in 129S6 Cdon mutant mice. t-BuOH is: 1) neither a substrate nor an inhibitor of ADHs or Cyp2E1; 2) poorly metabolized by oxidative processes; and 3) excreted mainly as the sulfate conjugate of the alcohol group [37–39]. Additionally, we tested the ability of antioxidants to influence EtOH-induced HPE in 129S6 Cdon mutant mice. We find that t-BuOH is a potent inducer of HPE, and that antioxidant treatment is not effective in preventing EtOH-induced HPE, in these mice. These findings are consistent with the notion ethanol itself, rather than a consequence of its metabolism, is an HPE-inducing teratogen.
Materials and methods

Mice

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee (IACUC). Our animal facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Two- to three-month-old Cdon<sup>+/tm1Rsk</sup> (Cdon<sup>+/−</sup>) mice on a 129S6/SvEvTac (129S6) background were mated for one hour in the dark and plugged females were collected. The time of the plug was designated as embryonic day (E) 0.0. EtOH administration was performed as described [25]. For t-BuOH treatment, pregnant female mice were injected intraperitoneally with 1g/kg of t-BuOH in saline at E7.0 and again 4 hours later. Saline injections were used as a control. We used the protocol described by Hirota et al. for antioxidant treatment [40]. Briefly, N-acetylcysteine (50 mg/kg body weight) and α-tocopherol (TCP, 1g/kg body weight) were given intraperitoneally at E5.0, E6.0 and E7.0. As an indicator of oxidative stress after alcohol treatment, total reactive oxygen species (ROS)/reactive nitrogen species (RNS) in the liver were analyzed with the OxiSelect In vitro ROS/RNS Assay Kit (Cell Biolabs) as per the manufacturer’s instructions. Briefly, the assay measures ROS/RNS-mediated formation of the fluorogenic product 2′,7′-dichlorodihydro-fluorescein (DCF) from a starting fluorogenic probe, 2′,7′-dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ). DCF fluorescence (λ<sub>ex</sub> = 480 nm, λ<sub>em</sub> = 530 nm) is proportional to the amount of ROS/RNS in the sample. Measurements were performed on a SpectraMax i3x microplate reader (Molecular Devices). Livers were homogenized on ice and centrifuged for 10,000g for 5 min. Protein concentrations were analyzed by Bradford assay. Liver GSH levels were measured using the GSH-Glo<sup>™</sup> Glutathione Assay (Promega) following the manufacturers protocol. Livers were harvested 12 hours after the initial dose of alcohol.

Histology and whole mount in situ hybridization

Embryos were dissected out and fixed overnight in 4% paraformaldehyde in PBS. They were then dehydrated through a graded ethanol series, embedded in paraffin and sectioned at 8 μm.
H&E staining was performed as described [25]. Slides were then dehydrated through graded ethanol and xylene and mounted with Permount (Fisher Scientific).

For whole-mount RNA in situ hybridization, E10.0 embryos were prepared essentially as described previously [41], except that they were treated with 10 μg/ml proteinase K (QIAGEN) in phosphate-buffered saline, 0.1% Tween-20 (PBT) for 45 minutes. Embryos were rinsed, postfixed, and hybridized with digoxigenin-labeled probe in hybridization mix [50% formamide, 1.3x SSC, 5 mM EDTA, 50 μg/ml yeast RNA, 0.2% Tween 20, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, and 100 μg/ml heparin] overnight at 65˚C. After washing and blocking, embryos were incubated overnight with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000; Roche) in blocking buffer (2% blocking reagent [Roche], 20% heat-inactivated lamb serum in 100 mM maleic acid, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 [MABT]). After washes in Tris-buffered saline with 0.1% Tween-20 (TBST) and 100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl2, and 0.1% Tween -20 (NTMT), signals were developed using BM Purple AP Substrate (Roche).

## Results

### t-BuOH induces HPE in Cdon mutant mice

To test whether oxidative catabolism is a critical feature of EtOH-induced HPE, we asked whether an alcohol congener not subject to such metabolism induces HPE in 129S6 Cdon mutant mice (hereafter simply called Cdon mutant mice). t-BuOH is not effectively metabolized by ADH or Cyp2E1 [37–39], so it was used in place of EtOH in our standard protocol [25]. Briefly, one-hour timed matings were set between Cdon heterozygotes, and pregnant females were treated IP with either t-BuOH in saline, or saline alone as a vehicle control, at E7.0 and again four hours later. A dose of 2 g/kg (t-BuOH/body weight) resulted in maternal lethality. A dose of 1 g/kg produced a spectrum of HPE phenotypes in Cdon-/- mice that was qualitatively and quantitatively similar to our standard protocol with EtOH (see below), and was not associated with any lethality, so this dose was used for detailed analysis. It should be noted that, on a moles-delivered basis, this dose of t-BuOH is ~18% that of EtOH in this model (1.0 vs. 3.48 g/kg, respectively, with the MW of t-BuOH = 74 and EtOH = 46).

Initial analyses were performed on embryos collected at E10.0. Similar to EtOH-treated embryos [25], t-BuOH-treated embryos had two or three fewer somite pairs at this stage than saline-treated controls, irrespective of genotype (Table 1). Therefore, as seen with in utero exposure to EtOH, t-BuOH caused a slight developmental delay that was independent of Cdon status. Approximately 15% of EtOH-treated Cdon-/- embryos displayed severe forms of HPE visible at E10.0 [25]. These embryos were characterized as having a small forebrain that failed

| Defect                  | Treatment | Genotype (# affected/total (%)) |
|-------------------------|-----------|---------------------------------|
|                         |           | Cdon<sup>+</sup> | Cdon<sup>-/-</sup> |
| External forebrain defects | Saline         | 0/12                | 0/10               |
|                         | t-BuOH       | 0/73                | 12/63 (19%)*       |
| # somites               | Saline         | 33.5 ± 1.45        | 33.5 ± 1.84        |
|                         | t-BuOH       | 30.48 ± 3.05**     | 31.0 ± 2.22**      |

*<p><0.0001 when comparing t-BuOH-treated Cdon<sup>-/-</sup> embryos with t-BuOH-treated Cdon<sup>+</sup> embryos with two-tailed Fisher’s exact test.

**<p><0.001 when comparing t-BuOH-treated embryos with saline-treated embryos of the same genotype using Student’s t-test. t-BuOH-treated embryos showed delayed somite formation irrespective of genotype.

https://doi.org/10.1371/journal.pone.0176440.t001
to partition, either fully or partially, into left and right hemispheres (these embryos died in utero and were resorbed before E12.0). A similar percentage (19%) of t-BuOH-treated Cdon<sup>−/−</sup> embryos also showed a severe forebrain HPE phenotype (Table 1, and see below). In contrast, wild type and Cdon<sup>+</sup> littermates were not affected by t-BuOH treatment, nor were saline-treated controls of any genotype.

We next collected and analyzed embryos at E14.0 to assess additional features of HPE, including midfacial and forebrain defects. Similar to what we have seen with EtOH, t-BuOH-treated Cdon<sup>−/−</sup> embryos developed externally visible HPE phenotypes, including fused upper lip and single nostril (arrow). (E-P) H&E stained coronal sections of E14 embryos. Midfacial and forebrain midline structures were missing or reduced in t-BuOH-treated Cdon<sup>−/−</sup> embryos, including cartilage primordium of the nasal septum (H, arrow); nasal septum (L, black arrow); vomeronasal organ (L, red arrow); defective palatal shelves (L, arrowheads) flanking midline cleft; and ventral diencephalon midline structure (P, arrow).

https://doi.org/10.1371/journal.pone.0176440.g002

| Cdon<sup>−/−</sup> | Cdon<sup>−/−</sup> |
|------------------|------------------|
| Saline | t-BuOH | Saline | t-BuOH |

**Fig 2.** t-BuOH induces HPE in Cdon mutant mice. (A-D) Frontal views of E14 embryos. t-BuOH-treated Cdon<sup>−/−</sup> embryos (D) developed strong facial features of HPE, including single nostril (arrow). (E-P) H&E stained coronal sections of E14 embryos. Midfacial and forebrain midline structures were missing or reduced in t-BuOH-treated Cdon<sup>−/−</sup> embryos, including cartilage primordium of the nasal septum (H, arrow); nasal septum (L, black arrow); vomeronasal organ (L, red arrow); defective palatal shelves (L, arrowheads) flanking midline cleft; and ventral diencephalon midline structure (P, arrow).
microphthalmia in one or both eyes (Fig 3, Table 2); these phenotypes were less common in EtOH-treated Cdon°/° embryos (~6% [25]).

Four E14.0 t-BuOH-treated Cdon°/° embryos with external HPE phenotypes and three embryos from each control group were sectioned and stained with Hematoxylin and Eosin (H&E). Lobar HPE, characterized by a partitioned forebrain that lacked ventral midline structure, was found in two out of four t-BuOH-treated Cdon°/° embryos (Fig 2, Table 2). All four t-BuOH-treated Cdon°/° embryos showed a narrower midface, lack of or diminished nasal septum, and defects in palate formation, including clefting (Fig 2, Table 2). These phenotypes and the frequency at which they were induced were very similar between EtOH- and t-BuOH-treated Cdon°/° embryos [25, 42]. t-BuOH-treated Cdon°/° embryos and saline-treated embryos of all genotypes showed normal developmental patterning of midline structures upon H&E staining (Fig 2, Table 2).

| Defect                      | Treatment | Genotype (# affected/total (%)) | Cdon°/° | Cdon°/° |
|-----------------------------|-----------|---------------------------------|---------|---------|
| Fused upper lip             | Saline    | 0/21                            | 0/29    |
|                             | t-BuOH    | 1/43 (2.3%)                     | 20/39 (51%)* |
| Single nostril              | Saline    | 0/21                            | 0/29    |
|                             | t-BuOH    | 1/43 (2.3%)                     | 7/39 (18%)** |
| Coloboma/microphthalmia     | Saline    | 0/21                            | 0/29    |
|                             | t-BuOH    | 0/43                            | 9/39 (23%)*** |
| Lobar HPE                   | Saline    | 0/3                             | 0/3     |
|                             | t-BuOH    | 0/3                            | 2/4²    |
| Defective palatogenesis     | Saline    | 0/3                             | 0/3     |
|                             | t-BuOH    | 0/3                            | 4/4²    |
| Diminished nasal septum     | Saline    | 0/3                             | 0/3     |
|                             | t-BuOH    | 0/3                            | 4/4²    |

*p<0.0001  
**p<0.05  
***p<0.001 when comparing t-BuOH-treated Cdon°/° embryos with saline-treated Cdon°/° embryos with two-tailed Fisher's exact test.  
¹These embryos did not have external HPE features.  
²These embryos displayed external HPE features.

https://doi.org/10.1371/journal.pone.0176440.t002

microphthalmia in one or both eyes (Fig 3, Table 2); these phenotypes were less common in EtOH-treated Cdon°/° embryos (~6% [25]).

Four E14.0 t-BuOH-treated Cdon°/° embryos with external HPE phenotypes and three embryos from each control group were sectioned and stained with Hematoxylin and Eosin (H&E). Lobar HPE, characterized by a partitioned forebrain that lacked ventral midline structure, was found in two out of four t-BuOH-treated Cdon°/° embryos (Fig 2, Table 2). All four t-BuOH-treated Cdon°/° embryos showed a narrower midface, lack of or diminished nasal septum, and defects in palate formation, including clefting (Fig 2, Table 2). These phenotypes and the frequency at which they were induced were very similar between EtOH- and t-BuOH-treated Cdon°/° embryos [25, 42]. t-BuOH-treated Cdon°/° embryos and saline-treated embryos of all genotypes showed normal developmental patterning of midline structures upon H&E staining (Fig 2, Table 2).

https://doi.org/10.1371/journal.pone.0176440.t002

**Fig 3. t-BuOH induces eye defects in Cdon mutant mice.** t-BuOH-treated Cdon°/° mice displayed microphthalmia and/or ventral coloboma (arrow), whereas t-BuOH-treated Cdon°/° mice and saline-treated mice of either genotype did not.

https://doi.org/10.1371/journal.pone.0176440.g003
Reduced expression of Shh and Nkx2.1 in the forebrains of t-BuOH-treated Cdon−/− embryos

Development of midline structures of the forebrain and midface is regulated by Shh pathway activity [43–48]. This occurs by a progressive mechanism, with a reiterative requirement for Shh signaling throughout rostroventral midline development, from early forebrain partitioning to fine patterning of the midface and palate; successful patterning of early midline structures is required for induction of Shh expression and pathway activation in midline structures that develop subsequently. The specificity of the Cdon mutation plus EtOH model, and the importance of Shh pathway signaling strength in EtOH-induced HPE, was demonstrated by the fact that removal of one copy of the negative regulator Ptc1 rescued HPE in >75% of treated Cdon−/− embryos [42]. Consistent with this notion, EtOH-treated Cdon−/− embryos display reduced expression of Shh and Shh pathway target genes at various stages of rostroventral midline development [25, 42]. To examine whether t-BuOH treatment also did so, we assessed expression of Shh and the direct Shh pathway target gene, Nkx2.1, in the developing forebrain by whole mount in situ hybridization of E10.25 embryos. Expression of both genes was decreased specifically in the ventral forebrains of t-BuOH-treated Cdon−/− embryos, but not any of the control embryos (Fig 4). As mentioned in the subsection above, we note that the t-BuOH-treated Cdon−/− embryo shown in panel 3H is an example of one with a very severe HPE phenotype.

Failure of antioxidants to rescue EtOH- or t-BuOH-induced HPE in Cdon−/− mice

Administration of exogenous antioxidant compounds can often ameliorate the effects of pro-oxidant toxins. We therefore tested whether this might be true for EtOH-induced HPE in Cdon−/− embryos. We selected an antioxidant regimen that had previously been demonstrated...
to be effective in reversing the effects in early pregnancy of the oxidant chemical, paraquat, in genetically-sensitized mice [40]. Pregnant females from intercrosses of Cdon+/− mice were administered IP a combination of N-acetylcysteine (50 mg/kg) and α-tocopherol (1 g/kg) on days E5.0, E6.0, and E7.0, plus or minus the standard E7.0 treatment with EtOH. Embryos were collected at E14.0 and scored for external signs of HPE, including fused upper lip, single nostril, and coloboma/microphthalmia. The frequencies of these EtOH-induced phenotypes were unchanged by antioxidant treatment (Fig 5, Table 3). Similarly, N-acetylcysteine/α-tocopherol administration did not prevent t-BuOH-induced HPE (Fig 5, Table 4).

To confirm that the antioxidant treatment relieved oxidant stress induced by EtOH, we employed a DCF fluorescence assay to measure levels of reactive oxygen and nitrogen species (ROS/RNS) in the livers of female mice 12 hours after treatment with EtOH. EtOH increased the levels of ROS/RNS by ~3.3-fold over that seen in saline-treated control mice (Fig 6). Co-administration of N-acetylcysteine/α-tocopherol with EtOH reduced these levels back to that seen in the controls. t-BuOH-treated mice also had significantly elevated levels of ROS/RNS (Fig 6). However, N-acetylcysteine/α-tocopherol administration was much less effective at normalizing ROS/RNS levels in t-BuOH-treated mice than in EtOH-treated animals. Although ROS/RNS levels trended lower in t-BuOH-treated mice co-administered the antioxidants, this was not statistically significant (Fig 6). We also measured levels of reduced glutathione (GSH) in livers of treated female mice. EtOH decreased GSH levels by ~60%, as compared to saline-treated mice (Fig 7). N-acetylcysteine/α-tocopherol administration increased GSH levels by ~50% in EtOH-treated mice, relative to EtOH treatment alone, whereas it did not have a significant effect on GSH levels in the saline-treated control mice (Fig 7). Again, results with GSH measurements in t-BuOH-treated mice differed from those with EtOH-treated mice (Fig 7). t-BuOH decreased GSH levels by ~40%, less than that seen with EtOH. In contrast to the findings with EtOH, N-acetylcysteine/α-tocopherol treatment had no effect on the t-BuOH-induced reduction in GSH levels. Therefore, N-acetylcysteine/α-tocopherol treatment re-normalized ROS/RNS levels and restored GSH levels to a significant extent, without altering the frequency of EtOH-induced HPE.

**Discussion**

EtOH is a human teratogen [15]. The range of effects in individuals exposed in utero to EtOH is broad and referred to under the umbrella term, fetal alcohol spectrum disorders (FASD). FASD can include a variety of structural and behavioral deficits [49]. Among the developmental defects that EtOH has been implicated in is HPE, the most common defect of forebrain and midface development [1]. Several mechanisms have been proposed to underlie EtOH’s varied effects on developing embryos.
teratogenic roles. Among these, some require its oxidative metabolism, while others do not. For example, metabolism by ADH and Cyp2E1 produces acetaldehyde, which is important for EtOH-induced exencephaly [28]. Additionally, it has been proposed that EtOH and acetaldehyde could act as competitive inhibitors during the enzymatic synthesis of RA from retinol (which involves ADH and ALDH reactions), resulting in developmental defects due to RA deficiency [29–31]. Moreover, EtOH metabolism leads to production of a variety of oxidant species, producing an overall condition of oxidative stress [26, 27]. On the other hand, EtOH itself has solvent properties and can perturb biological membranes [32–34]. EtOH also binds directly to the Ig cell adhesion molecule, L1, and interferes with its adhesive function [35, 36]. It seems likely that the multiple teratogenic outcomes associated with fetal alcohol exposure may be caused by distinct mechanisms, depending on the specific developmental defect in question.

To address how EtOH induces HPE, we used a mouse model that displays high specificity and fidelity to human HPE. Cdon mutation and in utero EtOH exposure synergize to produce a full range of HPE spectrum defects, with high penetrance [25]. We used this model to address the roles of EtOH metabolism and associated oxidative stress in HPE. We found that t-BuOH, a branched chain alcohol that is neither a substrate for, nor inhibitor of, ADH or Cyp2E1, was a potent inducer of HPE in Cdon−/− mice. Furthermore, t-BuOH induced a quantitatively and qualitatively similar response to EtOH at less than one-fifth the dose required for EtOH. These findings are consistent with the idea that it is EtOH itself, rather than its metabolism, which is required to induce HPE. One reason for t-BuOH’s greater potency is likely that, in the case of EtOH, the teratogen itself is depleted by ADH-dependent metabolism, whereas this does not occur with t-BuOH. t-BuOH is, however, metabolized by phase II enzymes and excreted (mainly as a sulfate conjugate), so it may also simply be a more efficient teratogen than EtOH. In addition to indicating that oxidative metabolism is probably unnecessary for

Table 3. Frequency of HPE defects in EtOH- plus antioxidant-treated mice at E14.

| Defect                  | Cdon+/− | Saline | EtOH | EtOH +NAC/TCP* | Cdon+/− | Saline | EtOH | EtOH +NAC/TCP* |
|-------------------------|---------|--------|------|---------------|---------|--------|------|---------------|
| Fused upper lip         |         | 0/48   | 0/33 | 0/10          |         | 0/22   | 1/22 | 13/18         | 15/20   |
| Single nostril          |         | 0/48   | 0/33 | 0/10          |         | 0/22   | 1/22 | 5/18          | 5/20    |
| Coloboma/ microphthalmia|         | 0/48   | 0/33 | 0/10          |         | 0/22   | 1/18 | 4/20          |

*NAC, N-acetylcysteine; TCP, α-tocopherol
**Frequencies of EtOH-induced HPE defects were not significantly altered by NAC/TCP.

https://doi.org/10.1371/journal.pone.0176440.t003

Table 4. Frequency of HPE defects in t-BuOH- plus antioxidant-treated mice at E14.

| Defect                  | Cdon+/− | t-BuOH | t-BuOH +NAC/TCP* | Cdon+/− | t-BuOH | t-BuOH +NAC/TCP* |
|-------------------------|---------|--------|------------------|---------|--------|------------------|
| Fused upper lip         |         | 1/43   | 0/12             |         | 20/39  | 8/15             |
| Single nostril          |         | 1/43   | 0/21             |         | 7/39   | 3/15             |
| Coloboma/ microphthalmia|         | 0/43   | 0/21             |         | 9/39   | 3/15             |

*NAC, N-acetylcysteine; TCP, α-tocopherol
**Frequencies of t-BuOH-induced HPE defects were not significantly altered by NAC/TCP.

https://doi.org/10.1371/journal.pone.0176440.t004
EtOH-induced HPE, our findings also suggest that competitive inhibition of retinol metabolism is not likely to be a major mechanism of EtOH-induced HPE either. It is worth noting in this regard that mice lacking \textit{RDH10} or \textit{Raldh2}, important enzymes in RA synthesis, have severe developmental defects but do not appear to have overt HPE [50–52]. Additionally, it has been concluded that the forebrain and facial defects seen in chick embryos treated with retinoid receptor antagonists are distinct from HPE [53].

\textbf{Fig 6. ROS/RNS levels in livers of mice from various treatment groups.} ROS/RNS levels were measured by production of the fluorescent compound, DCF. EtOH and \textit{t-BuOH} both increased ROS/RNS levels, relative to the saline control. N-acetylcysteine plus \alpha\-tocopherol (NAC+TCP) treatment normalized ROS/RNS levels in livers after EtOH exposure, but not after \textit{t-BuOH} exposure. *\textit{p}<0.05 with two-tailed Fisher’s exact test; n.s., not significant; values are means ± SD, n = 3–4 mice per point.

https://doi.org/10.1371/journal.pone.0176440.g006

\textbf{Fig 7. Reduced glutathione levels in livers of mice from various treatment groups.} (A, B) Reduced glutathione (GSH) levels were analyzed 12 hours after EtOH (A) or \textit{t-BuOH} (B) exposure. EtOH and \textit{t-BuOH} both decreased GSH levels, relative to the saline control. N-acetylcysteine plus \alpha\-tocopherol (NAC+TCP) treatment partially rescued GSH levels in livers after EtOH exposure (A), but not after \textit{t-BuOH} exposure (B). *\textit{p}<0.05 with two-tailed Fisher’s exact test; n.s., not significant; values are means ± SD, n = 3–4 mice per point.

https://doi.org/10.1371/journal.pone.0176440.g007
In a second approach, we found that administration of the antioxidants N-acetylcysteine/\( \alpha \)-tocopherol prior to and during EtOH or \( t \)-BuOH treatment did not alter the frequency or severity of HPE phenotypes. EtOH induced oxidative stress in mice, as evidenced by an increase in ROS/RNS levels and a reduction of GSH levels in the livers of treated female mice. N-acetylcysteine/\( \alpha \)-tocopherol treatment renormalized ROS/RNS levels, despite its lack of effect on EtOH-induced HPE. It also restored GSH levels, though not fully to the levels seen in untreated mice. These results argue that EtOH-induced oxidative stress can be segregated from its action as an HPE-inducing teratogen. It is difficult to accurately assess oxidative stress directly in the very early embryos that are exposed to EtOH, so we cannot fully rule out that the failure of antioxidant treatment to alter EtOH-induced HPE was due to an insufficient reversal of oxidant stress in the embryos themselves. Nevertheless, the EtOH-induced increase in liver ROS/RNS levels was returned to baseline with N-acetylcysteine/\( \alpha \)-tocopherol treatment. Furthermore, the antioxidant regimen used in these experiments was previously found to attenuate the effects of paraquat on embryo implantation in genetically sensitized mice [40]. Unlike EtOH, paraquat is thought to work exclusively via redox cycling and oxidative stress [54].

\( t \)-BuOH treatment also increased ROS/RNS levels and depressed GSH levels. However, administration of N-acetylcysteine/\( \alpha \)-tocopherol did not rescue ROS/RNS or GSH levels in \( t \)-BuOH-treated mice. Given that EtOH is oxidatively metabolized and \( t \)-BuOH is not, the mechanisms of EtOH- vs. \( t \)-BuOH-induced ROS/RNS production, GSH depletion, and, presumably, transient liver toxicity, may be different. It must be emphasized that despite these differences between EtOH and \( t \)-BuOH, each alcohol induced a very similar penetrance and spectrum of HPE phenotypes at the doses studied, arguing that the mechanism(s) that underlie their HPE teratogenicity are similar. Taking all the results together, we conclude that EtOH’s teratogenic effects in HPE are unlikely to involve oxidative metabolism and that oxidative stress itself may not be a critical component of its action in inducing HPE.

In utero EtOH exposure in C57BL/6J mice induces HPE with low penetrance [55, 56]. In contrast to our findings, Aoto et al. reported that feeding these mice a diet supplemented with \( \alpha \)-tocopherol prevented EtOH-induced HPE [55]. However, this system used very high levels of \( \alpha \)-tocopherol (5% of the diet). While \( \alpha \)-tocopherol has antioxidant properties, it may also function as a membrane stabilizer [57–59]. Given our findings in this study, we suggest that \( \alpha \)-tocopherol may have acted in this latter manner, at least in part, to prevent EtOH-induced HPE in C57BL/6J mice. It should be noted that prenatal antioxidant treatment is under consideration as a means of reversing or preventing EtOH’s teratogenicity in FASD [60]. Our results argue that this may be ineffective in HPE, and perhaps some other aspects of FASD.

If EtOH and \( t \)-BuOH are actual, rather than proximal, teratogens in HPE, what might be their mechanism of teratogenicity? EtOH is known to perturb membranes through solvent-like effects, and it seems likely that \( t \)-BuOH would be even more effective at this. Such a mechanism would also be consistent with the observation that high levels of \( \alpha \)-tocopherol prevented HPE when the combination of N-acetylcysteine/\( \alpha \)-tocopherol did not (albeit in different mouse strains). Membrane perturbation might in turn disrupt assembly or stability of membrane-associated signaling complexes that regulate rostroventral midline development during gastrulation, the time of sensitivity to EtOH-induced HPE. It is worth noting that the window of sensitivity to EtOH-induced HPE is very narrow, starting at about E7.0 and over by E7.5 ([25, 55] and our unpublished results). This is a time of rapid and extreme morphogenetic change that could be particularly susceptible to such effects. Finally, it is also possible that EtOH and \( t \)-BuOH could exert their effects via interaction with specific proteins, analogous to EtOH’s effects on the L1 cell adhesion molecule. It seems unlikely that L1 itself is the target in
HPE, however, as mutations in L1 in humans and mice are not known to be associated with HPE [61].

In summary, we report findings consistent with the notion that EtOH itself, rather than a consequence of its oxidative metabolism, acts as an HPE-inducing teratogen. We emphasize that other developmental defects associated with in utero EtOH exposure at different stages of development may arise from different mechanisms that do involve EtOH metabolism and oxidative stress [62, 63]. Use of a highly specific model of HPE allowed a specific focus on this defect, and it will be informative to apply these approaches to other systems with distinct EtOH-related outcomes.

Acknowledgments
We are grateful to Kuen Cheng for technical support and to Arthur Cederbaum and Paul Wassarman for helpful advice and discussions.

Author Contributions
Conceptualization: MH RSK.
Data curation: MH RSK.
Formal analysis: MH RSK.
Funding acquisition: RSK.
Investigation: MH.
Methodology: MH RSK.
Project administration: MH RSK.
Validation: MH RSK.
Visualization: MH RSK.
Writing – original draft: MH RSK.
Writing – review & editing: MH RSK.

References
1. Muenke M, Beachy PA. Holoprosencephaly. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The Metabolic & Molecular Bases of Inherited Disease. IV. Eighth Edition ed. New York: McGraw-Hill; 2001. p. 6203–30.
2. Shiota K, Yamada S. Early pathogenesis of holoprosencephaly. Am J Med Genet C Semin Med Genet. 2010; 154C:22–8. https://doi.org/10.1002/ajmg.c.30248 PMID: 20104600
3. Leoncini E, Baranello G, Orioli IM, Annerén G, Bakker M, Bianchi F, et al. Frequency of holoprosencephaly in the International Clearinghouse Birth Defects Surveillance Systems: searching for population variations. Birth Defects Res A Clin Mol Teratol. 2008; 82:585–91. https://doi.org/10.1002/bdra.20479 PMID: 18566978
4. Cohen MM Jr. Holoprosencephaly: clinical, anatomic, and molecular dimensions. Birth Defects Res Part A Clin Mol Teratol. 2006; 76:658–73. https://doi.org/10.1002/bdra.20295 PMID: 17001700
5. Krauss RS. Holoprosencephaly: new models, new insights. Expert Rev Mol Med. 2007; 9:1–17.
6. Solomon BD, Mercier S, Vélez JL, Pineda-Alvarez DE, Wylie A, Zhou N, et al. Analysis of genotype-phenotype correlations in human holoprosencephaly. Am J Med Genet C Semin Med Genet. 2010; 154C:133–41. https://doi.org/10.1002/ajmg.c.30240 PMID: 20104608
7. Wallis D, Muenke M. Mutations in holoprosencephaly. Hum Mutat. 2000; 16:99–108. https://doi.org/10.1002/1098-1004(200008)16:2<99::AID-HUMU2>3.0.CO;2-© PMID: 10923031
8. Johnson CY, Rasmussen SA. Non-genetic risk factors for holoprosencephaly. Am J Med Genet C Semin Med Genet. 2010; 154C:73–85. https://doi.org/10.1002/ajmg.c.30242 PMID: 20104598

9. Miller EA, Rasmussen SA, Siega-Riz AM, Frías JL, Honein MA. Study. NBDP. Risk factors for non-syndromic holoprosencephaly in the National Birth Defects Prevention Study. Am J Med Genet C Semin Med Genet. 2010; 154C:52–61. https://doi.org/10.1002/ajmg.c.30236 PMID: 20104595

10. Roessler E, Muenke M. The molecular genetics of holoprosencephaly. Am J Med Genet C Semin Med Genet. 2010; 154C:52–61. https://doi.org/10.1002/ajmg.c.30236 PMID: 20104595

11. Lacbawan F, Solomon BD, Roessler E, El-Jaick K, Domene S, Velez Ji, et al. Clinical spectrum of SIX3-associated mutations in holoprosencephaly: correlation between genotype, phenotype and function. Journal of Medical Genetics. 2009; 46(6):389–98. https://doi.org/10.1136/jmg.2008.063818 PMID: 19346217

12. Solomon B, Bear K, Wyllie A, Keaton A, Dubourg C, David V, et al. Genotypic and phenotypic analysis of 396 individuals with mutations in Sonic Hedgehog. Journal of Medical Genetics. 2012; 49(7):473–9. https://doi.org/10.1136/jmedgenet-2012-101008 PMID: 22791840

13. Roessler E, Velez JI, Zhou N, Muenke M. Utilizing prospective sequence analysis of SHH, ZIC2, SIX3 and TGIF in holoprosencephaly probands to describe the parameters limiting the observed frequency of mutant-gene-gene interactions. Mol Genet Metab. 2012; 105:658–64. https://doi.org/10.1016/j.ymgme.2012.01.005 PMID: 22310223

14. Chen JK. I only have eye for ewe: the discovery of cyclopamine and development of Hedgehog pathway-targeting drugs. Nat Prod Rep. 2016; 33:595–601. https://doi.org/10.1039/c5np00153f PMID: 26787175

15. Gilbert-Barness E. Teratogenic Causes of Malformations. Annals of Clinical & Laboratory Science. 2010; 40(2):99–114.

16. Krauss RS, Hong M. Gene–Environment Interactions and the Etiology of Birth Defects. Curr Top Dev Biol. 2016; 116:569–80. https://doi.org/10.1016/bs.ctdb.2015.12.010 PMID: 26970642

17. Lovely C, Rampersad M, Fernandes Y, J. E. Gene-environment interactions in development and disease. WIREs Dev Biol. 2016;

18. Bae GU, Domene S, Roessler E, Schachter K, Kang JS, Muenke M, et al. Mutations in CDON, Encoding a Hedgehog Receptor, Result in Holoprosencephaly and Defective Interactions with Other Hedgehog Receptors. Am J Hum Genet. 2011; 89:231–40. https://doi.org/10.1016/j.ajhg.2011.07.001 PMID: 21802063

19. Izzil L, Lévesque M, Morin S, Laniel D, Wilkes BC, Mille F, et al. Boc and Gas1 each form distinct Shh receptor complexes with Pcth1 and are required for Shh-mediated cell proliferation. Dev Cell. 2011; 20:788–801. https://doi.org/10.1016/j.devcel.2011.04.017 PMID: 21664577

20. Lu M, Krauss RS. N-cadherin ligation, but not Sonic hedgehog binding, initiates Cdo-dependent p38α/β MAPK signaling in skeletal myoblasts. Proc Natl Acad Sci (USA). 2010; 107:4212–7.

21. Cole F, Krauss RS. Microform holoprosencephaly in mice that lack the Ig superfamily member Cdon. Curr Biol. 2003; 13:411–5. PMID: 12620190

22. Zhang W, Kang J-S, Cole F, Yi MJ, Krauss RS. Cdo functions at multiple points in the Sonic Hedgehog pathway, and Cdo-deficient mice accurately model human holoprosencephaly. Dev Cell. 2006; 10:657–65. https://doi.org/10.1016/j.devcel.2006.04.005 PMID: 16647303

23. Allen BL, Tenzten T, McMahon AP. The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development. Genes Dev. 2007; 21:1244–57. https://doi.org/10.1101/gad.1543607 PMID: 17504941

24. Zhang W, Hong M, Bae G-U, Kang J-S, Krauss RS. Boc modifies the holoprosencephaly spectrum of Cdo mutant mice. Dis Model Mech. 2011; 4.

25. Hong M, Krauss RS. Cdon mutation and fetal ethanol exposure synergize to produce midline signaling defects and holoprosencephaly spectrum disorders in mice PLOS Genet. 2012; 8(10):e1002999. https://doi.org/10.1371/journal.pgen.1002999 PMID: 23071453

26. Cederbaum AI. Alcohol Metabolism. Clinics in Liver Disease. 2012; 16(4):667–85. https://doi.org/10.1016/j.cld.2012.08.002 PMID: 23101976

27. Heit C, Dong H, Chen Y, Shah Y, Thompson D, Vasiliev V. Transgenic Mouse Models for Alcohol Metabolism, Toxicity, and Cancer. In: Vasiliev V, Zakhari S, Seitz HK, Hoek JB, editors. Biological Basis of Alcohol-Induced Cancer. Advances in Experimental Medicine and Biology. 815: Springer International Publishing; 2015. p. 375–87.

28. Langevin F, Crossan GP, Rosado IV, Arends MJ, Patel KJ. Fancd2 counters the toxic effects of naturally produced aldehydes in mice. Nature. 2011; 475(7354):53–8. https://doi.org/10.1038/nature10192 PMID: 21734703
29. Deltour L, Ang HL, Duester G. Ethanol inhibition of retinoic acid synthesis as a potential mechanism for fetal alcohol syndrome. The FASEB Journal. 1996; 10(9):1050–7. PMID: 8801166

30. Kot-Leibovitch H, Fainsod A. Ethanol induces embryonic malformations by competing for retinaldehyde dehydrogenase activity during vertebrate gastrulation. Disease Models & Mechanisms. 2009; 2(5–6):295–305.

31. Yelin R, Kot H, Yelin D, Fainsod A. Early molecular effects of ethanol during vertebrate embryogenesis. Differentiation. 2007; 75(5):393–403. https://doi.org/10.1111/j.1432-0436.2006.00147.x PMID: 17286601

32. Goldstein DB. Effect of alcohol on cellular membranes. Annals of Emergency Medicine. 1986; 15(9):1013–8. PMID: 3526990

33. Klemm WR. Dehydration: A new alcohol theory. Alcohol. 1990; 7(1):49–59. PMID: 2178630

34. Lyon RC, McComb JA, Schreurs J, Goldstein DB. A relationship between alcohol intoxication and the disordering of brain membranes by a series of short-chain alcohols. Journal of Pharmacology and Experimental Therapeutics. 1981; 218(3):669–75. PMID: 7264950

35. Ramanathan R, Wilkemeyer MF, Mittal B, Perides G, Charness ME. Alcohol inhibits cell-cell adhesion mediated by human L1. The Journal of Cell Biology. 1996; 133(2):381–90. PMID: 8691700

36. Wilkemeyer MF, Menkari CE, Charness ME. Novel Antagonists of Alcohol Inhibition of L1-Mediated Cell Adhesion: Multiple Mechanisms of Action. Molecular Pharmacology. 2002; 62(5):1053–60. PMID: 12391267

37. Baker RC, Sorensen SM, Deitrich RA. The in vivo metabolism of tertiary butanol by adult rats. Alcohol Clin Exp Res. 1982; 6:247–51. PMID: 7048978

38. Beauté F, Clément M, Nordmann J, Nordmann R. Liver lipid disposal following t-butanol administration to rats. Chemo-Biological Interactions. 1981; 38(1):45–51. PMID: 7326806

39. Bernauer U, Amberg A, Scheutzow D, Dekant W. Biotransformation of 12C- and 2-13C-Labeled Methyl tert-Butyl Ether, Ethyl tert-Butyl Ether, and tert-Butyl Alcohol in Rats: Identification of Metabolites in Urine by 13C Nuclear Magnetic Resonance and Gas Chromatography/Mass Spectrometry. Chemical Research in Toxicology. 1998; 11(6):651–8. https://doi.org/10.1021/tx970215v PMID: 9625733

40. Hirota Y, Acar N, Tranchu S, Burnum KE, Xie H, Kodama A, et al. Uterine FK506-binding protein 52 (FKBP52)–peroxiredoxin-6 (PRDX6) signaling protects pregnancy from overt oxidative stress. Proc Natl Acad Sci USA. 2010; 107(35):15577–82. https://doi.org/10.1073/pnas.1009324107 PMID: 20713718

41. Mulieri PM, Kang J-S, Sassoon DA, Krauss RS. Expression of the boc gene during murine embryogenesis. Dev Dyn. 2002; 223:379–88. https://doi.org/10.1002/dvdy.10063 PMID: 11891987

42. Hong M, Krauss RS. Rescue of Holoprosencephaly in Fetal Alcohol-Exposed Cdon Mutant Mice by Reduced Gene Dosage of Ptch1. PLOS ONE. 2013; 8(11):e79269. https://doi.org/10.1371/journal.pone.0079269 PMID: 24244464

43. Aoto K, Shikata Y, Imai H, Matsumaru D, Tokunaga T, Shioda S, et al. Mouse Shh is required for pre-chordal plate maintenance during brain and craniofacial morphogenesis. Dev Biol. 2009; 327:106–20. https://doi.org/10.1016/j.ydbio.2008.11.022 PMID: 19103193

44. Cordero D, Marcucio R, Hu D, Gaffield W, Tapadia M, Helms JA. Temporal perturbations in sonic hedgehog signaling elicit the spectrum of holoprosencephaly phenotypes. J Clin Invest. 2004; 114:485–94. https://doi.org/10.1172/JCI19596 PMID: 15314685

45. Geng X, Speirs C, Lagutin O, Inbal A, Liu W, Solnica-Krezel L, et al. Haploinsufficiency of Six3 fails to activate Sonic hedgehog expression in the ventral forebrain and causes holoprosencephaly. Dev Cell. 2008; 15:236–47. https://doi.org/10.1016/j.devcel.2008.07.003 PMID: 18694563

46. Marcucio RS, Cordero DR, Hu D, Helms JA. Molecular interactions coordinating the development of the forebrain and face. Dev Biol. 2005; 284:48–61. https://doi.org/10.1016/j.ydbio.2005.04.030 PMID: 15979605

47. Marcucio RS, Young NM, Hu D, Hallgrimson B. Mechanisms that underlie co-variation of the brain and face. Genesis. 2011; 49:177–89. https://doi.org/10.1002/dvg.20710 PMID: 21381182

48. Schachter KA, Krauss RS. Murine models of holoprosencephaly. Curr Top Dev Biol. 2008; 84:139–70. https://doi.org/10.1016/S0070-2153(08)06003-0 PMID: 19186244

49. Riley EP, Inflante MA, Warren KR. Fetal Alcohol Spectrum Disorders: An Overview. Neuropsychol Rev. 2011; 21(2):73. https://doi.org/10.1007/s11065-011-9166-x PMID: 21499711

50. Niederreither K, Subbarayan V, Dolle P, Chambon P. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat Genet. 1999; 21(4):444–8. https://doi.org/10.1038/7788 PMID: 10192400
51. Ribes V, Wang Z, Dole P, Niederreither K. Retinaldehyde dehydrogenase 2 (RALDH2)-mediated retinoic acid synthesis regulates early mouse embryonic forebrain development by controlling FGF and sonic hedgehog signaling. Development. 2006; 133:351–61. https://doi.org/10.1242/dev.02204 PMID: 16368932

52. Sandell LL, Sanderson BW, Moiseyev G, Johnson T, Mushegian A, Young K, et al. RDH10 is essential for synthesis of embryonic retinoic acid and is required for limb, craniofacial, and organ development. Genes Dev. 2007; 21(9):1113–24. https://doi.org/10.1101/gad.1533407 PMID: 17473173

53. Schneider RA, Hu D, Rubenstein JL, Maden M, Helms JA. Local retinoid signaling coordinates forebrain and facial morphogenesis by maintaining FGF8 and SHH. Development. 2001; 128:2755–67. PMID: 11526081

54. Blanco-Ayala T, Andélica-Romero AC, Pedraza-Chaverri J. New insights into antioxidant strategies against paraquat toxicity. Free Radical Research. 2014; 48(6):623–40. https://doi.org/10.3109/10715762.2014.899694 PMID: 24593876

55. Aoto K, Shikata Y, Higashiyama D, Shiota K, Motoyama J. Fetal ethanol exposure activates protein kinase A and impairs Shh expression in prechondral mesendoderm cells in the pathogenesis of holoprosencephaly. Birth Defects Res A Clin Mol Teratol. 2008; 82:224–31. https://doi.org/10.1002/bdra.20447 PMID: 18338389

56. Kietzman HW, Everson JL, Sulik KK, Lipinski RJ. The teratogenic effects of prenatal ethanol exposure are exacerbated by Sonic Hedgehog or GLI2 haploinsufficiency in the mouse. PLOS ONE. 2014; 9(2): e89448. https://doi.org/10.1371/journal.pone.0089448 PMID: 24586787

57. Brigelius-Flóhe R. Vitamin E: The shrew waiting to be tamed. Free Radical Biology and Medicine. 2009; 46(5):543–54. https://doi.org/10.1016/j.freeradbiomed.2008.12.007 PMID: 19133328

58. Jurak M, Miñones Conde J. Characterization of the binary mixed monolayers of α-tocopherol with phospholipids at the air-water interface. Biochim Biophys Acta—Biomembranes. 2013; 1828(11):2410–8.

59. Quinn PJ. Is the distribution of alpha-tocopherol in membranes consistent with its putative functions? Biochemistry (Mosc). 2004; 69:58–66.

60. Gupta KK, Gupta VK, Shirasaka T. An Update on Fetal Alcohol Syndrome—Pathogenesis, Risks, and Treatment. Alcohol Clin Exp Res. 2016; 40(8):1594–602. https://doi.org/10.1111/acer.13135 PMID: 27375266

61. Schäfer MKE, Altevogt P. L1CAM malfunction in the nervous system and human carcinomas. Cellular and Molecular Life Sciences. 2010; 67(14):2425–37. https://doi.org/10.1007/s00018-010-0339-1 PMID: 20237819

62. Parnell SE, Sulik KK, Dehart DB, Chen S. Reduction of ethanol-induced ocular abnormalities in mice through dietary administration of N-acetylcysteine. Alcohol. 2010; 44(7–8):699–705. https://doi.org/10.1016/0.1016/j.alcohol.2010.05.006 PMID: 21112471

63. Wells PG, Bhatia S, Drake DM, Miller-Pinsler L. Fetal oxidative stress mechanisms of neurodevelopmental deficits and exacerbation by ethanol and methamphetamine. Birth Defects Res C Embryo Today. 2016; 108:108–30. https://doi.org/10.1002/bdrc.21134 PMID: 27345013