Table S1-1: Changes in DNA cytosine modifications and related enzymes following experimental *in utero* (not postnatal) alcohol exposure in the brain specifically.

| DNA cytosine modification | Result of ethanol exposure | Animal & Age at Sacrifice | Ethanol exposure | Brain region studied | Method | Reference |
|--------------------------|-----------------------------|---------------------------|------------------|----------------------|-------|----------|
| Promotor methylation of Slc6a4 | Higher Slc6a4 promoter CpG methylation at P55 | Rat; G21 and P55 | G1 - G21, ~12-15g ethanol per kg body weight per day | Hypothalamus | Bisulfite conversion, PCR and pyrosequencing | [5] |
| Methylation of CpG islands in Vmn2r64, Olf110, Olf601, Vpreb2 | Vmn2r64 hypermethylated, Olf110 hypo- and hypermethylated, Olf601 hypomethylated, Vpreb2 hypomethylated | Inbred mice; P28 and P60 | GD0.5 – 8.5, 12g±2.6g ethanol per kg body weight per day | P28: hippocampus | Bisulfite conversion, PCR and sequencing | [4] |
| CpG methylation of BDNF gene | increased CpG methylation | Sprague-Dawley rats | E1-E20, 6.0 g/kg alcohol daily by intragastric intubation | E21 granule cells of olfactory bulbs; P10 granule cells of olfactory bulbs | DNA digestion with methylation-sensitive enzyme *HpaII* followed by RT-PCR | [3] |
| CpG methylation of POMC gene | CpG methylation higher in F1 and F2 generations | Sprague-Dawley rats; both sexes | G7–G10 (1.7 to 5.0% v/v EtOH), then G11–G21 (6.7% v/v EtOH) (F1). FAE Offspring (F1) were bred again with controls on the opposite sex (F2). | Arcuate nucleus of the hypothalamus | DNA digestion with methylation-sensitive enzyme *HpaII* followed by RT-PCR; verified by pyrosequencing | [2] |
| Dnmt1 5hmC | increased Dnmt1, decreased 5hmC (mRNA), increased 5hmC (IF) | | | Arcuate nucleus of the hypothalamus, β-endorphin-positive cells | Immunofluorescence (IF); mRNA levels | |
| Dnmt1 Dnmt3a MeCP2 | Dnmt1 increased, Dnmt3a, increased (IF) but no change (mRNA), MeCP2 increased | Sprague-Dawley rats; males 60 to 65 days | G7 – G10 (1.7 to 5.0% v/v EtOH), then G11 – G21 (6.7% v/v EtOH) | β-endorphin-positive cells of the hypothalamus | mRNA levels & immunofluorescence | [1] |

G – gestational day, P – postnatal day, IF – immunofluorescence, CpG – Cytosine phosphate guanine, 5hmC – 5-hydroxymethylcytosine, Dnmt – DNA methyltransferase, MeCP2 – Methyl binding protein, BDNF – brain derived neurotropic factor, POMC – Proopiomelanocortin
Table S1-2: Histone post-translational modifications (PTMs) and enzymes associated with experimental in *utero* (not postnatal) alcohol exposure in the brain specifically

| Histone PTM | Result of Ethanol Exposure | Species & Age at Sacrifice | In utero ethanol exposure | Area Studied | Method | Reference |
|-------------|-----------------------------|-----------------------------|---------------------------|-------------|--------|-----------|
| H3K9ac      | decreased acetylation       | Sprague-Dawley rats; Males 60 to 65 days old | G7 – G10 (1.7 to 5.0% v/v EtOH), then G11 – G21 (6.7% v/v EtOH) | β-Endorphin-positive cells of the hypothalamus | Immuno-fluorescence | [1] |
| H3K9ac      | decreased acetylation       | Sprague-Dawley Rats; both sexes | G7 – G10 (1.7 to 5.0% v/v EtOH), then G11 – G21 (6.7% v/v EtOH) (F1). FAE Offspring (F1) were bred again with controls on the opposite sex (F2). | Arcuate of the hypothalamus β-Endorphin-positive cells | Immuno-fluorescence | [2] |
| H3K4me2, K4me3 | decrease methylation      | Sprague-Dawley rats; Males 60 to 65 days old | G7 – G10 (1.7 to 5.0% v/v EtOH), then G11 – G21 (6.7% v/v EtOH) | β-Endorphin-positive cells of the hypothalamus | Immuno-fluorescence | [1] |
| H3K4me2, K4me3 | decreased methylation      | Sprague-Dawley Rats; Both sexes 60 to 80 days old | G7 – G10 (1.7 to 5.0% v/v EtOH), then G11 – G21 (6.7% v/v EtOH) (F1). FAE Offspring (F1) were bred again with controls on the opposite sex (F2). | Arcuate of the hypothalamus β-Endorphin-positive cells | Immuno-fluorescence | [2] |
| H3K9me2     | increased methylation       | Sprague-Dawley rats; Males 60 to 65 days old | G7 – G10 (1.7 to 5.0% v/v EtOH), then G11 – G21 (6.7% v/v EtOH) | β-Endorphin-positive cells of the hypothalamus | Immuno-fluorescence | [1] |
| H3K9me2     | increased methylation       | Sprague-Dawley Rats; Both sexes 60 to 80 days old | G7 – G10 (1.7 to 5.0% v/v EtOH), then G11 – G21 (6.7% v/v EtOH) (F1). FAE Offspring (F1) were bred again with controls on the opposite sex (F2). | Arcuate of the hypothalamus β-Endorphin-positive cells | Immuno-fluorescence | [2] |
| Set7/9 G9a Setdb1 | Set7/9 decreased, G9a, increased, Setdb1 increased | Sprague-Dawley rats; males 60 to 65 days old | G7 – G10 (1.7 to 5.0% v/v EtOH), then G11 – G21 (6.7% v/v EtOH) | β-Endorphin-positive cells of the hypothalamus | mRNA levels | [1] |
| Set7/9 G9a Setdb1 Hdac2 | Set7/9 decreased, G9a, increased, Setdb1 increased Hdac2 increased | Sprague-Dawley Rats; Both sexes 60 to 80 days old | G7 – G10 (1.7 to 5.0% v/v EtOH), then G11 – G21 (6.7% v/v EtOH) (F1). FAE Offspring (F1) were bred again with controls on the opposite sex (F2). | Arcuate nucleus of hypothalamus | mRNA levels | [2] |

G – gestational day, Set – histone-lysine N-methyltransferase, G9a – Histone-lysine N-methyltransferase, HDAC – histone deacetylase
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Supplement 2.

Table S2 -1: Alcohol-exposed and control macaque details [1-3].

| ID number   | Sex | Age at sacrifice | Weekly dose of ethanol | Maternal peak plasma ethanol concentration | Abnormalities                                                                 | ID number   | Sex | Age at sacrifice |
|-------------|-----|------------------|------------------------|--------------------------------------------|-------------------------------------------------------------------------------|-------------|-----|------------------|
| 13077 / 29  | F   | 5.7 months       | 2.5g/kg                | 264mg/dl                                   | None                                                                          | 11023 / 2   | F   | 5.7 months       |
| 13076 / 28  | M   | 6.2 months       | 2.5g/kg                | 260mg/dl                                   | Strabismus                                                                    | 13071 / 4   | M   | 5.9 months       |
| 13078 / 30  | F   | 5.8 months       | 3.3g/kg                | 420mg/dl                                   | Significantly delayed learning, mild motor deficits                          | 10512 / 1   | F   | 5.8 months       |
| 13080 / 32  | F   | 6 months         | 3.3g/kg                | 432mg/dl                                   | Unusual auricles, metopic synostosis                                         | 10241 / 0   | F   | 6.3 months       |
| 13079 / 31  | M   | 6.1 months       | 3.3g/kg                | 432mg/dl                                   | Motor deficits                                                               | 13072 / 5   | M   | 6.1 months       |
| 11019 / 33  | M   | 7.2 months       | 4.1g/kg                | 540mg/dl                                   | Strabismus, bilateral microphthalmia, significant growth deficiency, microcephaly, motor deficits |             |     |                  |

Abbreviations: Female (F); Male (M); Grams per kilogram maternal bodyweight (g/kg); Milligrams per deciliter (mg/dl)
The 5 digit ID number represents the neuropathological ID and the 2 digit number represents the publication ID
PNAE macaques received ethanol (EtOH) during gestational weeks 5 – 24; Controls received isocaloric sucrose on weeks 1-24

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### Supplement 3:

Rationale for the selection of epigenetic marks (DNA cytosine modifications, histone acetylation and histone methylation) being investigated in PNAE human and macaque monkey brain samples. We wanted to select an assortment of epigenetic marks that had a variety of roles in the cell. The enzymes responsible for the addition and removal of these modifications are also listed. Lysine demethylase (KDM) and methyltransferase (KMT) aliases have been listed (a full comprehensive list can be found in Black et al. 2012 (Second reference)).

| Epigenetic mark | Role | Enzyme(s) responsible for modification | Removal of modification | Reference |
|-----------------|------|---------------------------------------|-------------------------|-----------|
| 5mC             | Gene repression and genomic imprinting. | DNMT1, DNMT3A, DNMT3B, DNMT3L | n/a         | [8–11]    |
| 5hmC            | Promotes gene expression during active demethylation. Associated with transcription. | TET1, TET2, TET3 | n/a |         |
| 5fC             | Enriched at poised enhancers and other regulatory elements. | TET1, TET2, TET3 | n/a |         |
| 5caC            | Along with 5fC, cause increased pausing, backtracking, and reduced fidelity of RNAPII. | TET1, TET2, TET3 | n/a |         |
| H3K4me3         | Associated with transcriptional start sites of actively transcribed genes. Enriched in gene promoters. Role in splicing. Regulates histone acetylation | SETD1A, SETD1B, PRDM9, MLL, MLL2-5, ASH1L | JARID1A-D, PHF8, JHDM1B, NO66 | [1–4, 13] |
| H3K9me2, K9me3 | Inactive gene promotors; associated with heterochromatin formation. | G9a, GLP, SETDB1, SETB2, SUV39H1, SUV39H2 | LSD1, JMJD2A-D |         |
| H3K27me2        | Silences enhancers. Transcriptionally silenced gene regions. Associated with heterochromatin. | G9a, GLP, EZH1, EZH2, NSD1, NSD3 | PHF8, JMJD3, UTX, JHDM1D |         |
| H3K27me3        | Inactive gene promotors. Enriched in gene promotors of developmentally regulated genes. Associated with facultative heterochromatin. Inhibits elongation. | EZH2, NSD3 | JMJD3, UTX |         |
| H3K36me3        | Transcriptionally active promotors. Associated with transcribed downstream gene regions. Promotes elongation. Role in splicing. Regulates histone acetylation | NSD1-3, ASH1L, SMYD2, SETMAR, SETD2 | JHDM1B, JMJD2A-D, NO66 |         |
| Histone Modification | Description | Associated Enzymes | Associated Proteins | References |
|----------------------|-------------|--------------------|---------------------|------------|
| H3K9ac               | Highly correlated with active promoters. Co-occurrence with H3K14ac and H3K4me3. Associated with active regulatory regions. | GCN5 (KAT2A), ELP3, CBP, P300 | SIRT1, SIRT6, Any HDAC | [1, 4–7, 12, 14] |
| H3K14ac              | Transcriptionally active chromatin/genes. | MYST3, GCN5 (KAT2A), CLOCK, NCOAT, GTF3C4 | SIRT1, Any HDAC | |
| H3K27ac              | Associated with active regulatory regions and enhancers. | CBP, P300 | Any HDAC | |
| H4K5ac               | Transcriptionally active regions. | Tip60, CBP, p300, KAT2A, KAT5, MYST2, HAT1 | Any HDAC | |
| H4K12ac              | Transcriptionally active promoters. | TIP60, HAT1, CBP, KAT2A, KAT5, MYST2, p300 | Any HDAC | |
| H4K16ac              | Gene promoters with the highest transcriptional activity as well as a proposed role in repression. High order chromatin structure. | MOF, CBP, KAT2A, p300, MYST1 | SIRT1, SIRT2, SIRT3, Any HDAC | |
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### Supplement 4: Procedural details of immunohistochemical labeling

| Antibody          | Antigen Retrieval | Dilution Human | Duration (hours) | DAB Time Human | Dilution Monkey | Duration (hours) | DAB Time Monkey | Antibody Diluent | Washing Buffer #1 | Washing Buffer #2 |
|-------------------|-------------------|----------------|-----------------|----------------|-----------------|-----------------|-----------------|-----------------|------------------|------------------|
| 5mC               | Sodium Citrate pH 6.0 | 1:500          | 1.5             | 5min           | 1:500           | 2               | 5min            | 1% BSA in PBS   | 1X PBS + 0.2% Triton-X-100 | PBS              |
| 5hmC              | Tris-EDTA pH 9.0   | 1:3000         | 1.5             | 5min           | 1:4000*         | 2               | 4min            | 1% BSA in TBS   | 1X TBS + 0.1% Tween 20 | TBS              |
| 5fC               | Tris-EDTA pH 9.0   | 1:400          | 1.5             | 6min           | 1:300           | 2.5             | 6min            | 1% BSA in TBS   | 1X TBS + 0.1% Tween 20 | TBS              |
| 5caC              | Tris-EDTA pH 9.0   | 1:250          | 1.5             | 6min           | 1:200           | 2.5             | 6min            | 1% BSA in TBS   | 1X TBS + 0.1% Tween 20 | TBS              |
| H3K4me3           | Sodium Citrate pH 6.0 | 1:500          | 2               | 5min           | 1:200           | 2               | 6min            | 1% BSA in PBS   | 1X PBS + 0.2% Triton-X-100 | PBS              |
| H3K9ac            | Tris-EDTA pH 9.0   | 1:500          | 1.5             | 6min           | 1:300           | 2               | 6min            | 1% BSA in TBS   | 1X TBS + 0.1% Tween 20 | TBS              |
| H3K9me2, K9me3**  | Sodium Citrate pH 6.0 | 1:250          | n/a             | 1:250          | 2.5             | 5min            | 1% BSA in PBS   | 1X PBS + 0.2% Triton-X-100 | PBS              |
| H3K14ac           | Sodium Citrate pH 6.0 | 1:150          | 1.5             | 5min           | n/a             |                 |                 | 1% BSA in PBS   | 1X PBS + 0.2% Triton-X-100 | PBS              |
| H3K27ac           | Sodium Citrate pH 6.0 | 1:500          | 1.5             | 5min           | 1:400           | 2               | 5min            | 1% BSA in PBS   | 1X PBS + 0.2% Triton-X-100 | PBS              |
| H3K27me3          | Sodium Citrate pH 6.0 | 1:150          | 1.5             | 5min           | 1:100           | 2.5             | 6min            | 1% BSA in PBS   | 1X PBS + 0.2% Triton-X-100 | PBS              |
| H3K36me3          | Tris-EDTA pH 9.0   | 1:100          | 1.5             | 5mins          | 1:250*          | 2               | 5min            | 1% BSA in TBS   | 1X TBS + 0.1% Tween 20 | TBS              |
| H4K5ac            | Tris-EDTA pH 9.0   | 1:350          | 1.5             | 5min           | 1:250           | 2               | 5min            | 1% BSA in TBS   | 1X TBS + 0.1% Tween 20 | TBS              |
| H4K12ac           | Tris-EDTA pH 9.0   | 1:250          | 1.5             | 5min           | 1:100           | 2               | 5min            | 1% BSA in TBS   | 1X TBS + 0.1% Tween 20 | TBS              |
| H4K16ac           | Sodium Citrate pH 6.0 | 1:100          | 1.5             | 6min           | n/a             |                 |                 | 1% BSA in PBS   | 1X PBS + 0.2% Triton-X-100 | PBS              |
| H3panAc           | Sodium Citrate pH 6.0 | 1:200          | 1.5             | 5min           | 1:150           | 2               | 6min            | 1% BSA in PBS   | 1X PBS + 0.2% Triton-X-100 | PBS              |
| H4panAc           | Sodium Citrate pH 6.0 | 1:150          | 2               | 5min           | n/a             |                 |                 | 1% BSA in PBS   | 1X PBS + 0.2% Triton-X-100 | PBS              |
| Total H3          | Sodium Citrate pH 6.0 | 1:300          | 1.5             | 6min           | 1:75            | 3               | 6min            | 1% BSA in PBS   | 1X PBS + 0.2% Triton-X-100 | PBS              |
| Total H4          | Tris-EDTA pH 9.0   | 1:1000         | 1.5             | 5min           | 1:800           | 2               | 5min            | 1% BSA in TBS   | 1X TBS + 0.1% Tween 20 | TBS              |
* The concentrations of the supplied lots differed between human and monkey experiments

** The epigenetic mark H3K9me2/K9me3 was assessed in the macaques but this antibody was discontinued by the manufacturer and was not available for the human cohort. We purchased anti-H3K9me2 monoclonal antibody from the same manufacturer (#ab176882, Abcam) as an alternative. Despite being reported for use in immunohistochemistry, we did not succeed in getting it to work in the human PNAE samples after multiple attempts.

Note: among the macaques, anti-H3K14ac and anti-H4K16ac did not work for immunohistochemistry.

Abbreviations: BSA – bovine serum albumin; PBS - phosphate-buffered saline; TBS - Tris-buffered saline; min -minutes
Supplement 5

Figure S5-1: Representative immunohistochemical detection of epigenetic marks in human control temporal neocortex showing discrepancies between selective and total histone antibodies. Red arrows depict the difference in intensity and blue arrows point at negative nuclei primarily in the background. Antibodies to total histone H3, total histone H4, H3 pan acetyl, and H4 pan acetyl do not necessarily show more widespread immunoreactivity than corresponding more selective antibodies to H3K27ac and H4K12ac. Images were taken at 400x magnifications. DAB detection of antibody (brown) and hematoxylin counterstain (blue).
Figure S5-2: Representative immunohistochemical detection of epigenetic mark H3K36me3 in human control ventricular (VZ) and subventricular zones (SVZ). In the VZ, the control case demonstrates strong immunoreactivity, decreases, then increases again ('U' shape) before its disappearance at 31-34 weeks post-conception. Immunoreactivity in the SVZ increased with increasing age. Images were taken at 200x magnification. DAB detection of antibody (brown) and hematoxylin counterstain (blue). The age in post-conception weeks (w) is indicated for each panel.
Figure S5-3: Representative immunohistochemical detection of epigenetic marks in human control vascular endothelial cells, arterial smooth muscle cells lining blood vessels, and epithelial cells of the choroid plexus. a) H3K9ac (41 post-conception weeks (w)). b) 5mC (20w). c) H3K27ac (43w). d) H4K5ac (57.5w). e) H3K4me3 (66w). f) H3K36me3 (66w). Note that the endothelial and smooth muscle nuclei are typically a mix of positive and negative, while the choroid plexus cells tend to be more uniformly positive. Images were taken at 400x magnification. DAB detection of antibody (brown) and hematoxylin counterstain (blue).
Supplement 6: Proportion rank values representing specific brain cell types among the temporal ependyma, dentate gyrus, temporal cortex and white matter in human control fetal and infant cases for each of the epigenetic marks studied.

| Epigenetic Mark | Temporal Ependyma | Dentate Gyrus | CA1 | Temporal Cortex, Layer 5/6 | White Matter, near cortical layer 6 |
|-----------------|------------------|--------------|-----|---------------------------|----------------------------------|
|                 | Epithelial       | Granular     | Pyramidal | Large Neuron | Astrocyte or Interneuron | Oligodendrocyte | Microglia | Endothelial/Smooth Muscle | Large Neuron* | Astrocyte or Interneuron | Oligodendrocyte | Microglia | Endothelial/Smooth Muscle |
| FETUS ~38-40 week post-conception |
| 5mC             | 2                | 2            | 3       | 4            | 3                        | 1           | 1          | 2          | 4 | 3 | 2 | 1 | 2 |
| 5hmC            | 4                | 3            | 3       | 4            | 3                        | 3           | 2          | 1          | 4 | 2 | 1 | 2 | 2 |
| 5fC             | 1                | 2            | 1       | 0            | 0                        | 0           | 0          | 0          | 0 | 0 | 0 | 0 | 0 |
| 5caC            | 2                | 2            | 2       | 0            | 1                        | 0           | 0          | 0          | 0 | 1 | 1 | 1 | 0 |
| H3K4me3         | 3                | 3            | 3       | 4            | 3                        | 2           | 2          | 1          | 3 | 3 | 2 | 2 | 2 |
| H3K27me3        | 2                | 2            | 3       | 4            | 3                        | 1           | 1          | 1          | 4 | 3 | 2 | 2 | 1 |
| H3K36me3        | 4                | 3            | 4       | 4            | 4                        | 3           | 3          | 2          | 3 | 2 | 3 | 3 | 2 |
| H3K9ac          | 2                | 2            | 3       | 2            | 2                        | 1           | 0          | 0          | 1 | 1 | 1 | 0 | 0 |
| H3K14ac         | 2                | 2            | 2       | 0            | 1                        | 1           | 0          | 1          | 0 | 0 | 1 | 0 | 1 |
| H3K27ac         | 4                | 4            | 4       | 4            | 4                        | 3           | 3          | 2          | 4 | 3 | 3 | 3 | 2 |
| H4K5ac          | 4                | 4            | 4       | 4            | 4                        | 3           | 3          | 2          | 4 | 4 | 3 | 3 | 2 |
| H4K12ac         | 3                | 3            | 4       | 4            | 4                        | 3           | 3          | 1          | 4 | 4 | 3 | 3 | 1 |
| H4K16ac         | 1                | 2            | 1       | 0            | 0                        | 0           | 0          | 0          | 0 | 0 | 0 | 0 | 0 |
| INFANT ~53-57.5 week post-conception |
| 5mC             | 3                | 4            | 4       | 4            | 4                        | 3           | 1          | 1          | 4 | 4 | 3 | 3 | 1 |
| 5hmC            | 4                | 3            | 3       | 4            | 3                        | 2           | 1          | 1          | 4 | 2 | 2 | 1 | 1 |
| 5fC             | 4                | 2            | 2       | 0            | 2                        | 3           | 1          | 1          | 1 | 4 | 3 | 3 | 2 |
| 5caC            | 4                | 3            | 2       | 1            | 3                        | 3           | 2          | 1          | 2 | 4 | 4 | 3 | 3 |
| H3K4me3         | 3                | 3            | 4       | 4            | 3                        | 2           | 2          | 1          | 4 | 4 | 2 | 2 | 1 |
| H3K27me3        | 3                | 3            | 3       | 4            | 3                        | 2           | 1          | 1          | 4 | 2 | 1 | 1 | 1 |
| H3K36me3        | 4                | 4            | 4       | 4            | 4                        | 3           | 4          | 3          | 4 | 4 | 4 | 3 | 3 |
| H3K9ac          | 3                | 3            | 4       | 3            | 3                        | 2           | 2          | 1          | 4 | 3 | 3 | 3 | 1 |
| H3K14ac         | 4                | 3            | 3       | 2            | 3                        | 2           | 1          | 1          | 4 | 3 | 2 | 2 | 1 |
| H3K27ac         | 3                | 3            | 4       | 4            | 3                        | 3           | 1          | 0          | 4 | 2 | 2 | 2 | 2 |
| H4K5ac          | 4                | 4            | 4       | 4            | 4                        | 3           | 3          | 2          | 4 | 4 | 4 | 3 | 3 |
| H4K12ac         | 4                | 4            | 4       | 4            | 4                        | 3           | 3          | 2          | 4 | 4 | 2 | 3 | 2 |
| H4K16ac         | 2                | 4            | 4       | 3            | 3                        | 3           | 1          | 0          | 4 | 2 | 2 | 2 | 0 |

4 = ~100% positive  3 = ~75% positive  2 = ~50% positive  1 = ~25% positive  0 = ~0% positive
*white matter neurons
Supplement 7.
Representative photomicrographs showing immunohistochemical detection of 5fC and H3K27me3 epigenetic marks in control and PNAE macaque temporal horn ependyma. Compared to controls, the decrease among PNAE for 5fC (p=0.0077) and H3K36me3 (p=0.0058) were statistically significant. For 5caC (\( \downarrow p=0.0210 \)) and H3K9ac (\( \downarrow p=0.0427 \)), trends were noted (images not shown). Prenatal alcohol exposure is also shown in g/kg maternal body weight. Images were taken at 400x magnification. DAB detection of antibody (brown) and hematoxylin counterstain (blue).
Supplement 8

Lack of immunohistochemical evidence for oxidative damage in human and monkey brains with prenatal alcohol exposure.

Introduction

Reactive oxygen species (ROS; which include superoxide anion, hydroxyl radical, and hydrogen peroxide) along with reactive nitrogen species (RNS; which include nitric oxide and peroxinitrite) are important byproducts of brain metabolism [5, 8]. Under normal physiological conditions, antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) detoxify and maintain the proper redox potential in the cells. External factors such as alcohol consumption and cigarette smoking can lead to an increase in the level of ROS, which can cause damage to macromolecules including DNA, proteins, and lipids. Oxidative damage is postulated in the pathogenesis of brain damage in fetal alcohol spectrum disorder (FASD) and has been demonstrated in a variety of experimental situations [2, 18, 19].

We hypothesized that markers of oxidative stress will be evident in brains of human fetuses and neonates with documented prenatal alcohol exposure (PNAE)[9] and in brains of 6 month macaque monkeys that had been exposed to weekly high doses of alcohol during the fetal period [4]. We focused on the youngest human cohorts to avoid detection of changes incurred during postnatal life.

Methods

Detection of oxidative stress damage

Twelve human PNAE cases (6 fetuses, 6 infants) were selected from our previously published cohort [9] according to tissue preservation and availability of temporal lobe / hippocampus samples. Controls were matched for age, sex, and death circumstances (Table 1). Fetuses were stillborn, but had only minimal evidence of skin maceration and brain autolysis. Infant cases were all sudden deaths (typically while sleeping). Most had brief attempts at resuscitation. Autopsies were performed 1-2 days after delivery (fetuses) or death (infants), the brains were fixed in 10% buffered formalin for 10-20 days, and samples of brain were embedded in paraffin.

Samples of medial temporal lobe including hippocampus were selected for evaluation. On hematoxylin and eosin stain, none had histologic evidence of malformation. Some of the fetal control and PNAE cases had scattered karyorrhectic neurons indicative of in utero hypoxic damage to neurons.
None of the infant had histologic evidence for acute or remote hypoxic-ischemic neuronal injury or for infection.

Table 1: Human control and PNAE cases matched for age and sex.

| Group       | Pair  | Control Age (a) | PNAE Age | Sex |
|-------------|-------|-----------------|----------|-----|
| Fetuses     | 1     | 22 weeks        | 23 weeks | M   |
|             | 2     | 37              | 36       | M   |
|             | 3     | 38              | 36       | F   |
|             | 4     | 38              | 40       | F   |
|             | 5     | 38              | 40       | F   |
|             | 6     | 40              | 40       | M   |
| Infants     | 7     | 24 days         | 23 days  | F   |
|             | 8     | 2 months        | 2 months | M   |
|             | 9     | 3               | 3        | M   |
|             | 10    | 5               | 5        | F   |
|             | 11    | 7               | 7        | M   |
|             | 12    | 9               | 11       | F   |

Notes: a) Fetal ages are expressed as gestational weeks. Infant ages are expressed as postnatal months unless otherwise shown as days.

Paraffin sections (5 µm thick) were subjected to immunohistochemical staining to detect markers of oxidation. Glutamate cysteine ligase catalytic (GCLC) subunit is critical for synthesis of glutathione, one of the regulators of cellular redox state. In chronic or repeated oxidative challenge, GCLC may be upregulated as a compensatory response [15]. Malondialdehyde (MDA) is a product of cell lipid peroxidation [7, 16]. Another aldehyde formed during lipid peroxidation is 4-hydroxynonenal (HNE), which reacts to form stable adducts with proteins and DNA [1]. Oxidative damage to DNA can be
detected more directly by immunostaining for 8-hydroxy-deoxyguanosine (8OHdG) [13]. Previously validated, commercially available antibodies (Table 2) were optimized for immunohistochemistry using positive control tissues with probable oxidative damage (Alzheimer disease brain, subacute ischemic brain damage, metastatic adenocarcinoma to brain, and normal colon).

Table 2: Oxidative marker antibodies; conditions used for immunohistochemistry

| Name                                      | Antibody type       | Source and Catalogue # | Antibody concentration (mg / mL) | Secondary detection       |
|-------------------------------------------|---------------------|------------------------|----------------------------------|---------------------------|
| Glutamate cysteine ligase catalytic (GCLC)| Rabbit polyclonal   | Abcam (ab40929)        | 0.00125                          | Goat Anti-Rabbit          |
| Malondialdehyde (MDA)                    | Rabbit polyclonal   | Abcam (ab6463)         | 0.0006667                        | Goat Anti-Rabbit          |
| 4 Hydroxynonenal (4HNE)                  | Rabbit polyclonal   | Calbiochem (393207)    | 0.0006667                        | Goat Anti-Rabbit          |
| 8-Hydroxy-deoxyguanosine (8OHdG)         | Mouse monoclonal    | Abcam (ab48508)        | 0.02                             | Goat Anti-Mouse           |

Slides were dewaxed in xylene and rehydrated, then antigen retrieval was performed by heating in sodium citrate buffer (pH6) in a pressure steamer (95-100°C) for 15 minutes. Nonspecific peroxidase was inactivated by immersion in 3% hydrogen peroxide in methanol for 10 minutes followed by blocking with 1.5% goat serum in phosphate buffered saline (PBS) for 30 minutes. Primary antibodies were diluted in PBS at a range of concentrations bracketing the manufacturers’ suggested dilution and applied to the slide for 90 minutes. After washing, slides were incubated with biotinylated secondary antibody (1/500 dilution) for 30 minutes, then streptavidin-peroxidase conjugate for 60 minutes. Bound antibody was detected with 10% diaminobenzidne in hydrogen peroxide for 10 minutes, after which slides were counterstained with hematoxylin and a coverslip was affixed.

After optimal immunostaining conditions were established using the positive control samples, the same conditions were used for control and PNAE brain slides. Temporal cortex and hippocampi were examined at 100x and 400x magnifications, with specific attention to cell types that can be
identified unambiguously (e.g. large neurons, endothelial cells). A blinded paired evaluation was used to
determine if the oxidative marker was similar or differed between the controls and exposed subjects.
Intensity and distribution of immunolabeling was blindly categorized into four ordinal variables (0=
absent, 1= low, 2= moderate, and 3= high). Ordinal data allow only non-parametric statistical
comparisons. We tested the hypothesis that PNAE fetuses and / or infants have a greater burden of
oxidative markers (i.e. a one-tailed test). For the human data, we used a matched pairs Wilcoxon signed
rank test (nonparametric equivalent of a paired t test). For the monkey data we used a Kruskal-Wallis 2-
sample test (JMP 14.2 software, SAS Institute).

Results

GCLC immunoreactivity was moderately intense in the cytoplasm of human white matter glial
cells (usually large cells with the morphology of myelination glia) in 2/5 near-full term fetal controls
(Figure 1A) and in medium-size cortical cells (likely neurons) in 1/6 control cases. Weak
immunoreactivity in similar cells was also observed. Paired comparisons showed that human PNAE
cases tended to have less GCLC immunolabeling than controls (p=0.938 for fetuses and p=0.500 for
infants). In the monkey brains, moderate GCLC immunoreactivity was evident in the ependyma, choroid
plexus, and glia limitans. There was no difference between groups (p=0.204).

4HNE immunoreactivity was evident in the white matter glial cells of most human fetuses
(Figure 1B), but negligible labeling was evident in human infant brains. Paired comparisons showed that
human PNAE cases tended to have less 4HNE immunolabeling than controls (p=0.500 for fetuses and
p=0.500 for infants). No 4HNE immunolabeling was apparent in any of the monkey brains; it is not
clear if this reflects a true absence or a failure to detect because of overfixation.

Moderate MDA immunoreactivity was evident in the rare scattered neurons of most human
fetuses (Figure 1C), but only weak, inconsistent ependymal labeling was evident in human infant brains.
Paired comparisons showed that human PNAE cases tended to have less MDA immunolabeling than
controls (p=0.875 for fetuses and p=0.750 for infants). No MDA immunolabeling was apparent in any of
the monkey brains; it is not clear if this reflects a true absence or a failure to detect because of
overfixation.

Strong nuclear 8OHDG immunolabeling could be demonstrated in rare cells of the positive
control tissue, including Alzheimer disease brain (not shown). However, under identical fixation and
processing conditions no labeling was evident in human fetal or infant brains except for weak focal
labeling in some subpial glial cells of a single infant. Paired comparisons showed no difference between human control and PNAE cases (p=0.625 for fetuses and p=0.500 for infants). In one control and one PNAE monkey, a subset of arachnoid cell nuclei was labeled for 8OHDG (Figure 1D) (p=1.000).

Discussion

This study shows some evidence for MDA in neurons, along with 4HNE and GCLC in white matter glia of stillborn fetuses. This might simply reflect the hypoxic stress that leads to in utero death of the fetus. Among the markers evaluated by immunostaining, none demonstrated increased oxidative stress in the in utero alcohol exposed fetuses or infants. Previous studies of rat and mouse embryonic brains have shown increased levels of oxidative markers soon after maternal administration of ethanol. These include 8OHDG [3, 6], 4HNE [14], and GCLC [12]. There are several possible explanations for discrepancies between the rodent and primate findings: 1) Oxidative changes in the brain might be a species-specific effect found in PNAE rodents but not primates. At a pure cell toxicity level this seems unlikely, but placental biology is known to differ [10]. 2) The in utero alcohol exposure might not have been sufficient in the humans and monkeys. As previously noted, in humans the true exposure is very difficult to ascertain [9]; however, these monkeys were previously shown to have a FASD-like phenotype [4]. 3) Post exposure in utero conditions or postnatal life circumstances in monkeys and humans might allow gradual elimination of the markers of in utero hypoxic stress [11]. We cannot know when the most recent alcohol exposure was in the fetuses, and we know for certain that the human and monkey infants had at least several weeks to months to compensate for the alcohol exposure. 4) The circumstances leading to in utero demise of the human fetuses are associated with oxidative changes [17]. These might obscure relatively subtle changes associated with PNAE.

The major limitation of studying human brain tissues is that the details of PNAE (quantity, frequency of alcohol, and time of exposure) cannot be known with certainty. Further, we cannot be absolutely certain that the control samples did not also have PNAE, which was not elucidated in the history. The binge-exposure macaque brain tissues offer control of the exposure.

In summary, immunohistochemical detection of oxidative changes in protein, lipid, and DNA did not demonstrate evidence for PNAE-associated oxidative damage in the human (fetal and infant) or monkey (infant) brain. However, we cannot conclude that this mechanism does not transiently contribute to the pathophysiology of PNAE-associated brain changes.
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Figure legend
A. Glutamate cysteine ligase catalytic (GCLC) immunoreactivity (brown; arrows) in the cytoplasm of temporal white matter glial cells in a 38 gestational week stillborn control fetus. Original magnification 600x; hematoxylin counterstain (blue nuclei).

B. 4 Hydroxynonenal (4HNE) immunoreactivity (brown; arrows) in the cytoplasm of temporal white matter glial cells in a 38 gestational week stillborn control fetus. Original magnification 400x; hematoxylin counterstain (blue nuclei).

C. Malondialdehyde (MDA) immunoreactivity (brown; arrows) in the cytoplasm of temporal cortex neurons of a 40 gestational week stillborn control fetus. Original magnification 400x; hematoxylin counterstain (blue nuclei).

D. 8-Hydroxy-deoxyguanosine (8OHDG) immunoreactivity (brown; arrows) in the nuclei of arachnoidal cells of a 6 month in utero alcohol-exposed monkey. Original magnification 600x; hematoxylin counterstain (blue nuclei).
