Low-Dose Thyroxine Attenuates Autism-Associated Adverse Effects of Fetal Alcohol in Male Offspring’s Social Behavior and Hippocampal Gene Expression

Elif Tunc-Ozcan*, Timothy M. Ullmann*, Pradeep K. Shukla, and Eva E. Redei

**Background:** Fetal alcohol spectrum disorder (FASD) is characterized by neurodevelopmental anomalies manifesting in cognitive and behavioral deficits in the offspring with diverse severities. Social behavior is affected in FASD, and these deficits overlap with those of autism spectrum disorder (ASD). Identifying some of the molecular characteristics related to ASD in an animal model of FASD could ultimately provide details on the underlying molecular mechanisms of both disorders that could lead to novel treatments.

**Methods:** Pregnant Sprague-Dawley rats received the following diets: control (C; ad libitum standard laboratory chow), nutritional control pair-fed (PF), ethanol (EtOH), or an EtOH diet supplemented with 0.3, 1.5, or 7.5 mg thyroxine (T4)/l in the diet. Social behavior and memory were tested in the adult offspring. Plasma total T4, free T3 (fT3), and thyroid-stimulating hormone (TSH) levels were measured. Hippocampal expression of Gabrb3, Ube3a, Nr2b, Rasgrf1, and Dio3 were measured by RT-qPCR and protein levels of Mecp2 and Slc25a12 by Western blotting.

**Results:** Adult male offspring of EtOH dams showed elevated fT3 and low TSH levels. Adult male, but not female, offspring of EtOH dams exhibited social behavior and memory deficits. Expression of autism candidates, Gabrb3, Ube3a, Mecp2, and Slc25a12, was significantly increased in the hippocampus of male offspring of EtOH dams. Hippocampal Nr2b and Dio3 were also increased, while Rasgrf1 was decreased in the same population. Peripheral thyroid function, social behavioral deficits, and altered expression of the above genes were normalized by simultaneous administration of 0.3 mg/l T4 in the EtOH diet.

**Conclusions:** Our data suggest that social interaction deficits of FASD share molecular mechanism with ASD by showing altered hippocampal expression of several ASD candidate genes. Social interaction deficits as well as the gene expression changes in the offspring of EtOH-consuming dams can be reversed by low dose of thyroid hormone supplementation to the mothers.

**Key Words:** Fetal Alcohol Spectrum Disorders, Autism Spectrum Disorders, Social Interaction, Social Memory, Thyroxine.
evidence from both human and animal studies that autism is caused by insults to the developing fetus in utero (Arndt et al., 2005; Bishop et al., 2007; Rinaldi et al., 2007). The fetus is most sensitive to the majority of these insults during the first trimester, which corresponds to the peak period of ethanol (EtOH) vulnerability (Arndt et al., 2005). Despite the similarities in the etiologies and presentations of ASD and FASD, Bishop and colleagues (2007) demonstrated that in humans there are subtle, yet key differences between the typical phenotypes of the 2 families of disorders. Therefore, identifying some of the molecular characteristics related to ASD in an animal model of FASD could ultimately provide details on the underlying molecular mechanisms of both disorders that could lead to novel treatments.

In this study, we aimed to examine the expression of hippocampal genes associated with the genetics and pathophysiology of ASD in the offspring of EtOH-consuming dams. These genes include ubiquitin protein ligase E3A (Ube3a) and Gabbr3, the gene coding for the β3 subunit of the GABA<sub>A</sub> receptor (Samaco et al., 2005), which are mapped to chromosome 15q11–13 that is the most commonly reported genetic loci implicated in autism in human (Cook et al., 1997; Hogart et al., 2007). Expression of the ASD-related methyl CpG-binding protein 2 (Mecp2; Samaco et al., 2005) and Slc25a12, a mitochondrial glutamate/aspartate carrier protein (Lepagnol-Bestel et al., 2008), was also explored in the hippocampus of EtOH offspring. Additionally, we investigated the changes of NMDA receptor subunit 2b (Nr2b) and ras guanine nucleotide releasing factor 1 (Rasgrf1) expression in response to prenatal EtOH exposure. Nr2b has been implicated in the pathophysiology of ASD (Choudhury et al., 2012) as well as of FASD (Samudio-Ruiz et al., 2010), and Rasgrf1 has been shown to be involved in Nr2b-dependent processes that are important for hippocampal-dependent memory (Giese et al., 2001). Additionally, a recent study reports that structural variation in deiodinase-3 opposite strand, Dio3os, which is coregulated with Dio3 (Dietz et al., 2012), is involved in ASD (Matsunami et al., 2013). As we have shown that hippocampal expression of this thyroid hormone metabolizing enzyme, Dio3, is functionally associated with decreased olfactory investigation of the EtOH male offspring in the social interaction test (Sittig and Redei, 2011; Sittig et al., 2011b), we also investigated whether thyroxine (T4) administration would affect expression differences of Dio3 in this study.

In our previous study, we reported that administration of a pharmacological dose of T4 to the EtOH-consuming pregnant dams reversed some of the cognitive deficits seen in EtOH offspring (Wilcoxon et al., 2005), but suppressed plasma thyroid-stimulating hormone (TSH) and free T3 (FT3) in the female EtOH offspring (Wilcoxon and Redei, 2004). As intrauterine thyroid dysfunction has also been hypothesized to contribute to autism (Sadamatsu et al., 2006), in this study, we examined the effects of lower doses of T4 supplementation to the EtOH-consuming dams on the social deficits of their offspring and on the selected hippocampal mediators of ASD.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Northwestern University Animal Care and Use Committee. Maternal diet and animal procedures were performed as described previously (Wilcoxon et al., 2005). Shortly, adult female Sprague-Dawley (SD) rats (Harlan, Indianapolis, IN) were mated with SD males overnight, and gestational day 1 (G1) was assigned by the presence of sperm in vaginal smears. Pregnant females were assigned to 1 of 6 diet groups on G4, control (C; ad libitum standard laboratory chow), pair-fed (PF), EtOH, or an EtOH diet supplemented with 1 of 3 doses of T4. All pregnant rats except those assigned to the control group received ad libitum isocaloric liquid diet (Lieber-DeCarli '82) from G4 to G8. On G8, rats started their assigned diet. EtOH dams received a diet consisting of 5% w/v, 35% EtOH-derived calories. PF dams received an amount of isocaloric liquid diet that matched the paired EtOH dam’s diet consumption in the previous day. The 3 T4 groups received the EtOH diet that was additionally supplemented with 0.3 (EtOH + T4 [0.3]), 1.5 (EtOH + T4 [1.5]), or 7.5 (EtOH + T4 [7.5]) mg T4/l diet. The EtOH diet was introduced in stages starting on G8 with one-third EtOH diet and two-thirds standard liquid diet, followed by two-thirds EtOH and one-third standard diet, and from G10 to G21 EtOH rats received a full 5% (w/v) EtOH diet. The 3 T4 groups received the T4-supplemented EtOH diet starting on G8 as described above. Liquid diets were replaced with laboratory chow on G21. Number of litters for each group: C = 5, PF = 5, EtOH = 5, EtOH + T4 (0.3) = 4, EtOH + T4 (1.5) = 4, and EtOH + T4 (7.5) = 4. Blood alcohol concentrations were not measured in this study, but EtOH diet consumption was similar to those observed in the same animal model previously (Sittig and Redei, 2010) and resulted in blood alcohol levels of 126 mg/dl.

Pups were weaned at 24 days of age and housed separately by sex and treatment. All adult rat offspring underwent behavioral testing at approximately the same age. Only 1 to 2 animal/sex/litter were used for the different testing.

Two weeks following the completion of behavioral testing, adult rat offspring were sacrificed by decapitation between 10:00 and 12:00 am. Trunk blood was collected into EDTA-coated tubes on ice, and plasma was obtained by centrifugation. Frontal cortex (A/P 5.2-2.7; M/L 0-3.3; D/V 9.0-5.0; Paxinos Brain Atlas) and whole hippocampus were immediately dissected and collected directly into RNALater reagent (Ambion, Austin, TX) or on dry ice and stored at −80°C.

Social Interaction Test

Social interaction test was performed as described previously (Sittig et al., 2011b). At approximately 70 days of age, rats were isolated in a clean cage for 24 hours. Following the isolation period, an unrelated and unfamiliar male rat pup (pup 1) aged about 28 days was placed into the cage. The animals’ behavior was recorded on video for 4 minutes, after which time pup 1 was removed from the cage, and the adult rat allowed to rest for 1 hour. Following the hour-long break, pup 1 was reintroduced into the cage along with a second, unfamiliar pup (pup 2). The animals’ behavior was again recorded for 4 minutes before the pups were removed. Following the experiment, the adult rats were returned to their original cages with their cage mates. At a later date, a trained observer scored olfactory investigation of the adult toward each pup, defined as seconds spent in sniffing the familiar or novel pup.

RNA Isolation and Quantitative RT-PCR

Total RNA extraction, reverse transcription (RT), and quantitative PCR (qPCR) were performed as described previously (Shukla et al., 2011). Briefly, RNA was isolated using Trizol reagent.
according to the manufacturer’s instructions (Life Technologies, Grand Island, NY). RT of 1 μg total RNA was performed using the TaqMan Reverse Transcription kit (Applied Biosystems, Branchburg, NJ). Real-time PCR was conducted with the ABI 7300 system using SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Reactions were performed in triplicate and reached threshold amplification within 32 PCR cycles. Relative levels of gene expressions were determined relative to 18S (primers commercially available from ABI, Foster City, CA) and a general calibrator using the 2−ΔΔCt method. The primer pairs used for each gene are listed in Table 1.

Protein Isolation and Western Blot

Hippocampus of adult offspring was individually homogenized in ice-cold lysis buffer as described previously (Shukla et al., 2010). Samples containing 60 μg protein were electrophoresed on either 12 or 7.5% (w/v) SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes for detection with MeCP2 and Slc25a12 primary antibodies (Abcam, Cambridge, MA). Membranes were incubated overnight at 4°C in a primary antibody concentration of 1:1,000 in 5% milk (w/v) in blocking buffer. The membranes were then incubated in 1:1,000 secondary antibody solutions for 2 hours and 30 minutes, and then developed using Amer sham ECL Plus Western Blotting System (GE Healthcare, Little Chalfont, UK). β-actin protein levels were measured in the same membranes using a monoclonal antibody (1:10,000; Sigma, St. Louis, MO). The optical density of each protein was normalized to the corresponding β-actin signal using ImageJ software (NIH, Bethesda, MD).

Radioimmunoassays

Total T4 and fT3 as well as TSH in plasma were measured by RIA as previously described (Sittig and Reide, 2010). A rat TSH RIA manufactured by Alpco Diagnostics (Salem, NH) was used according to manufacturer’s instructions with assay sensitivity 1.6 ng/ml and intra-assay coefficients of variation (CV) 3.9%. Total T4 and fT3 assays were manufactured by MP Biomedicals, LLC (Irvine, CA) and assay sensitivity/CVs were T4: 0.8 μg/dl, 3.3%; fT3: 0.6 μg/ml, 8%.

RESULTS

Morphometric Measurements

Based on the diet consumption, the 3 T4 groups received the EtOH diet that was additionally supplemented with 0.3 (EtOH + T4 [0.3]), 1.5 (EtOH + T4 [1.5]), or 7.5 (EtOH + T4 [7.5]) mg T4/l of diet received increasing doses of T4 (Table 2). Dams on the 2 higher T4 doses (EtOH + T4 [1.5] and EtOH + T4 [7.5]) consumed significantly less liquid diet than all the other diet-fed animals, F(4, 21) = 25.8, p < 0.01 (Table 2). Subsequently, they did not receive the same dose of EtOH as the dams fed an EtOH or EtOH + T4 (0.3) diet, F(4, 16) = 9.0, p < 0.01 (Table 2). Although the thyroid functions of these offspring were tested, they were excluded from subsequent studies.

All mothers, regardless of diet, gave birth to litters of approximately the same size and sex ratio (Table 3). There were no visible morphological changes in the newborns, as expected from this moderate EtOH consumption paradigm. Pup weights were recorded at weaning to not disturb animals while nursing. Pup weights did not vary by sex or by prenatal diet, except that pups of the EtOH + T4 (0.3) mothers weighed less than pups of all other dams, F(5, 42) = 3.29, p < 0.05 (Table 3).

As expected, adults males weight significantly more than females, F(1, 85) = 1,613.0, p < 0.01 (Table 4). The only adult weight difference due to prenatal diet was that male offspring of EtOH + T4 (1.5) dams weighed significantly more than rats from diet groups C, PF, and EtOH + T4 (0.3), F(5, 85) = 2.33, p < 0.05 (Table 4). Adrenal weights were normalized to body weight and showed a signi-

**Table 1.** Quantitative RT-PCR Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nr2b</td>
<td>F: GGACCTCGGATATTCCACAC&lt;br&gt;R: CAGCTGCTCGCTCTTTGTT</td>
</tr>
<tr>
<td>Ube3a</td>
<td>F: TGGAAAACGGAAGAAATTTTGTCA&lt;br&gt;R: CGTGGAAATGCTCTTTGTT</td>
</tr>
<tr>
<td>Gabrb3</td>
<td>F: AGCAAGATATTGGAGGCTCTCT&lt;br&gt;R: CGATCCAAAGATTTCTAGTGAAGATGT</td>
</tr>
<tr>
<td>Rasgrf1</td>
<td>F: AGAGGAGCTGTCGCCAGGTCAT&lt;br&gt;R: CCTCGGTGAATCAGTGCAAA</td>
</tr>
<tr>
<td>Dio3</td>
<td>F: CTTTGCAGGCGCTTCTCTA&lt;br&gt;R: GTCCCTTGTCCGTAGCAGG</td>
</tr>
</tbody>
</table>

**Table 2.** Morphometric Measurement and Proportion of Liquid Diet, Ethanol (EtOH), and T4 Consumption by Maternal Diet

<table>
<thead>
<tr>
<th>Maternal diet</th>
<th>G21 matenal weight (g)</th>
<th>Average liquid consumed (m/l/d)</th>
<th>EtOH consumption (mg/100 g BW/d)</th>
<th>T4 dose (mg/100 g BW/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>315 ± 3.9</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PF</td>
<td>313 ± 3.9</td>
<td>78.4 ± 0.4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EtOH + T4 (0.3)</td>
<td>314 ± 1.6</td>
<td>76.6 ± 0.6</td>
<td>135.8 ± 2.3</td>
<td>0.8 ± 0.0</td>
</tr>
<tr>
<td>EtOH + T4 (1.5)</td>
<td>327 ± 7.9</td>
<td>70.1 ± 0.7</td>
<td>113.7 ± 5.2</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>EtOH + T4 (7.5)</td>
<td>309 ± 3.1</td>
<td>69.3 ± 0.3*</td>
<td>120.9 ± 4.5*</td>
<td>18.0 ± 0.8</td>
</tr>
</tbody>
</table>

C, control; PF, pair-fed; T4, thyroxine; BW, body weight. *p < 0.01 compared with liquid diet consumed by all other groups. †p < 0.01 compared with EtOH and EtOH + T4 (0.3).

Values are shown as mean ± SEM.

**Table 3.** Litter Sizes, Male/Female Ratios, and Weaning Weights by Sex Obtained from Different Diet Groups

<table>
<thead>
<tr>
<th>Maternal diet</th>
<th>Litter size</th>
<th>Male/ female ratio</th>
<th>Male pups weight (g)</th>
<th>Female pups weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>10.4 ± 0.5</td>
<td>1.0 ± 0.19</td>
<td>34.4 ± 0.8</td>
<td>34.3 ± 1.4</td>
</tr>
<tr>
<td>PF</td>
<td>14.2 ± 0.4</td>
<td>1.0 ± 0.16</td>
<td>31.8 ± 1.0</td>
<td>33.6 ± 0.9</td>
</tr>
<tr>
<td>EtOH</td>
<td>11.0 ± 1.6</td>
<td>1.5 ± 0.63</td>
<td>36.3 ± 2.8</td>
<td>35.6 ± 3.4</td>
</tr>
<tr>
<td>EtOH + T4 (0.3)</td>
<td>12.8 ± 1.7</td>
<td>1.0 ± 0.31</td>
<td>28.5 ± 0.2*</td>
<td>30.3 ± 1.0*</td>
</tr>
<tr>
<td>EtOH + T4 (1.5)</td>
<td>9.3 ± 1.9</td>
<td>1.4 ± 0.85</td>
<td>33.1 ± 1.2</td>
<td>34.2 ± 2.4</td>
</tr>
<tr>
<td>EtOH + T4 (7.5)</td>
<td>9.5 ± 2.5</td>
<td>2.0 ± 0.56</td>
<td>32.8 ± 1.2</td>
<td>32.8 ± 1.1</td>
</tr>
</tbody>
</table>

EtOH, ethanol; C, control; PF, pair-fed; T4, thyroxine. *p < 0.05 compared with all other groups. Values are shown as mean ± SEM.
Table 4. Body and Adrenal Weights of Adult Offspring by Maternal Diet and Sex

<table>
<thead>
<tr>
<th>Maternal diet</th>
<th>Sex</th>
<th>Body weight (g)</th>
<th>Adrenal weight (mg)/BW(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>M</td>
<td>394 ± 10.0</td>
<td>0.194 ± 0.017</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>238 ± 3.4</td>
<td>0.294 ± 0.015</td>
</tr>
<tr>
<td>PF</td>
<td>M</td>
<td>389 ± 5.2</td>
<td>0.167 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>250 ± 5.2</td>
<td>0.388 ± 0.022</td>
</tr>
<tr>
<td>EtOH</td>
<td>M</td>
<td>406 ± 5.1</td>
<td>0.215 ± 0.014</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>243 ± 4.2</td>
<td>0.349 ± 0.006</td>
</tr>
<tr>
<td>EtOH + T4 (0.3)</td>
<td>M</td>
<td>384 ± 11.8</td>
<td>0.207 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>249 ± 4.7</td>
<td>0.315 ± 0.020</td>
</tr>
<tr>
<td>EtOH + T4 (1.5)</td>
<td>M</td>
<td>420 ± 10.1145</td>
<td>0.226 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>251 ± 6.8</td>
<td>0.361 ± 0.012</td>
</tr>
<tr>
<td>EtOH + T4 (7.5)</td>
<td>M</td>
<td>408 ± 6.9</td>
<td>0.165 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>246 ± 4.7</td>
<td>0.286 ± 0.017</td>
</tr>
</tbody>
</table>

EtOH, ethanol; C, control; PF, pair-fed; T4, thyroxine; BW, body weight. *p < 0.05 compared with EtOH. †p < 0.01, ‡p < 0.05 compared with C. §p < 0.05 compared with PF. *p < 0.05 compared with EtOH + T4 (0.3).

Values are shown as mean ± SEM.

Table 4. Body and Adrenal Weights of Adult Offspring by Maternal Diet and Sex

The first trial of the social interaction test measures the social interest and curiosity of the test animal, by how much time the adult rat spends in olfactory investigation of the pup. Male and female offspring showed significantly different levels of social interaction, and prenatal treatment had a significant, but differential effect on male and female offspring (Fig. 2A, diet: F(3, 43) = 3.10, p < 0.05; sex: F(1, 43) = 10.29, p < 0.01; sex × diet: F(3, 43) = 45.52, p < 0.01). Specifically, male rats prenatally exposed to EtOH showed significantly decreased olfactory investigation compared with rats whose mothers consumed a C diet (Fig. 2A, p < 0.05). However, when EtOH-consuming mothers’ diets were supplemented with 0.3 mg T4/l diet, this social deficit disappeared which was displayed as a significant (p < 0.01) increase in olfactory investigation compared with EtOH animals. In contrast, adult female offspring of dams on different diets exhibit significantly increased social interaction compared with controls (Fig. S2A).

The second half of the social interaction test employs the familiar pup from trial 1 (T1) and a novel pup. The total time spent with olfactory investigation of these 2 pups presented a profile similar to the first test; EtOH offspring exhibited decreased olfactory investigation compared with PF (Fig. 2B, p < 0.01), which was reversed by prenatal T4 treatment, F(3, 23) = 10.32, p < 0.01 (EtOH vs. EtOH + T4, **p < 0.01).
THYROXINE ATTENUATES FETAL ALCOHOL EFFECTS

Fig. 2. Adult male offspring of ethanol-consuming mothers (E) show social interaction (A and B) and social memory deficit (C), these deficits are attenuated by administration of 0.3 mg T4/l in the EtOH-containing liquid diet (EtOH + T4 [0.3]). (A) Social interaction is measured by time spent (in seconds) with olfactory investigation of an unfamiliar test pup in the first trial of the social interaction test. (B) Social interaction is measured by total time spent (in seconds) with olfactory investigation of the familiar test pup from the first trial and an unfamiliar test pup introduced in second part of the social interaction test. (C) Social memory is characterized by the time difference between investigating the same test pup in the first and the second trials (T1–T2 in seconds). Details are as in Fig. 1. n = 7/group.

Altered Expression of Hippocampal Genes After Prenatal Exposure to EtOH

Hippocampal gene expression of selected genes was measured by RT-qPCR, shown in Fig. 3. Transcript levels were measured in males only, because only male offspring of EtOH mothers demonstrated a behavioral deficit in the social interaction test.

Levels of transcripts for autism candidate genes, Gabrb3 and Ube3a, were significantly increased in the hippocampus of adult male offspring of EtOH dams compared with those of C, but not PF, Gabrb3: F(3, 12) = 4.18, p = 0.05; Ube3a: F(3, 12) = 6.81, p = 0.05 (C vs. EtOH, p = 0.05; Fig. 3A,B). Transcript levels of NMDA receptor subunit Nr2b were similarly increased in the male EtOH hippocampus as well as in the PF hippocampus compared with C, F(3, 12) = 14.58, p < 0.01 (Fig. 3C; C vs. EtOH, p < 0.01; C vs. PF, p < 0.05). In contrast, mRNA levels of Rasgrf1 were decreased by prenatal EtOH treatment, F(3, 13) = 6.17, p < 0.01 (Fig. 3D). There was a similar effect of low-dose T4 administration (EtOH + T4 [0.3]) for the reversal of prenatal EtOH-induced changes in the expression of these genes, which reached significance for Rasgrf1 (C vs. EtOH, p < 0.01; EtOH vs. EtOH + T4, p < 0.05). Dio3 expression was significantly increased in the hippocampus of EtOH animals compared with both C and PF groups (C vs. EtOH, p < 0.05; PF vs. EtOH, p < 0.05), which was normalized in the EtOH + T4 (0.3) group, F(3, 14) = 7.74, p < 0.01 (Fig. 3E; EtOH vs. EtOH + T4, p < 0.01). Frontal cortex Dio3 expression has been shown not to be altered by prenatal alcohol exposure (Sittig et al., 2011b). Nr2b expression was not measured in the frontal cortex. There were no significant differences in the transcript levels of Gabrb3, Ube3a, and Rasgrf1 in frontal cortex of adult male offspring from different prenatal treatment groups (Fig. S3).

Elevated Hippocampal MeCP2 and Slc25a12 Protein Levels After Prenatal Exposure to EtOH

Protein levels of both MeCP2 and Slc25a12 increased significantly in the hippocampus of adult male offspring of EtOH dams compared with C, MeCP2: F(3, 16) = 4.25, p < 0.05; Slc25a12: F(3, 18) = 4.39, p < 0.05 (Fig. 4). The low-dose T4 administration concomitantly with EtOH attenuated this increase for both proteins, MeCP2: t(7) = 1.97, p = 0.08; Slc25a12: t(6) = 1.47, p = 0.09. There were no significant differences in the protein levels of MeCP2 and Slc25a12 in the frontal cortex of adult male offspring from different prenatal treatment groups (Fig. S4).

Thyroid Hormone Responsive Elements in Genes Associated with ASD

As altered expressions of ASD-related genes in the hippocampus of EtOH offspring were reversed by prenatal T4 treatment of the alcohol-consuming dams, we investi-
gated the presence of Thyroid Hormone Responsive Elements (TREs) in the promoter regions (2,000 bp upstream of the start site) of these genes. TRE sites were predicted using Transcription Element Search System (http://www.cbil.upenn.edu/tess). TREs were found in all, except Dio3, of both human and rat genes as shown in Table 5.

**DISCUSSION**

There are 2 major findings of this study. First, our data suggest that social interaction deficits caused by prenatal EtOH exposure share molecular mechanisms with ASD by showing altered hippocampal expression of several ASD
candidate genes. Second, we demonstrated that social interaction deficits as well as prenatal EtOH exposure-induced changes in hippocampal gene expression in the offspring of EtOH-consuming dams can be reversed by low dose of thyroid hormone supplementation to the EtOH-consuming pregnant dams. Although these results are novel and can lead to interesting hypotheses, the behavioral and gene expression findings are correlational; therefore, their causal connection will need to be investigated in the future.

Social Behavior Deficits

FAE caused decreased social interaction in the adult male, but not female, SD rats compared with offspring of controls. This male-specific finding is robust as it is in agreement with previous reports across several laboratories and different paradigms (Hamilton et al., 2010; Kelly, 1996; Kelly and Dillingham, 1994; Kelly et al., 2000, 2009; Sittig et al., 2011b). In contrast to the deficit in social behavior in males, female offspring of EtOH-consuming dams showed increased social interaction and memory. This prenatal EtOH-induced enhanced social interaction has been previously shown in female rats (Hamilton et al., 2010; Kelly and Dillingham, 1994). Although there are no sex differences explicitly reported in the social behavior of children and adolescents with FASD, gender differences in other behavioral and cognitive outcomes of FASD are well known (Herman et al., 2008). It is interesting to note that there is also a sex bias in the incidence of ASD, such as autism is 4 times more common in males than in females (Baron-Cohen et al., 2009).

The cause of this profound sex difference in social interaction deficit of EtOH offspring is not known. If this sex difference has similar molecular causality to the sex differences in the prevalence of ASD, changes in fetal or perinatal levels of testosterone could be involved. However, while some evidence links elevated fetal testosterone levels to autistic symptomatology (Auyeung et al., 2010), both fetal and neonatal testosterone levels are decreased in male offspring of EtOH-consuming rat dams (McGivern et al., 1988). It is a possibility, though, that positive or negative alterations from normal fetal testosterone levels could lead to the observed sex differences in the behavioral measures. A potential alternative explanation for these behavioral differences proposes that short periods of social isolation can alter the central mechanism of social behavior system through changes in social motivation and possibly through changes in social learning (Lugo et al., 2003). In our study, rats were socially isolated for 24 hours that could be stressful and reported sex differences in social behavior may reflect the differential sensitivity to such a stressor between male and female rats (Palanza, 2001).

Gene Expression Changes

The findings that several ASD-related genes showed hippocampal expression changes in male EtOH offspring are of great interest, as it poses the hypothesis of common molecular mechanism(s) between the autism- and prenatal EtOH-induced social interaction deficits (Olexova et al., 2012). However, expression of UBE3A and GABRB3 is decreased in autism brain samples (Hogart et al., 2007), in contrast to the increased expression found here in the EtOH male hippocampus. The concurrence of increased protein levels of Mecp2 with the increased transcript levels of hippocampal Ube3a and Gabrb3 is exactly the opposite that was found in ASD (Samaco et al., 2005). These 3 opposite directional changes in EtOH male hippocampus are in contrast to findings in ASD and are reminiscent of the fetal testosterone differences in FASD models and those proposed in ASD. Protein levels of another autism candidate gene, Slc25a12, were also increased in the EtOH male hippocampus, but this increase is similar to what is found in autism brain samples (Palmieri et al., 2010). Aralar, which is another alias of Slc25a12, is an aspartate/glutamate carrier, and increased plasma levels of glutamate has been found in autism that correlates with levels of social impairment (Shinohe et al., 2006). Thus, increased protein levels of Slc25a12 in the EtOH male hippocampus may contribute to the defined social behavioral deficit.

Our finding of increased Nr2b expression in the EtOH male hippocampus has not resolved the contradiction in the literature regarding the direction of change in Nr2b expression levels by prenatal alcohol (Samudio-Ruiz et al., 2010). The elevated Nr2b expression in the PF hippocampus indicates that nutritional deficit in the EtOH group may also contribute to the increased Nr2b expression in the EtOH hippocampus. However, this increased Nr2b expression is similar to the significantly increased Nr2b expression in an animal model of autism, rats prenatally exposed to valproic acid.
acid (Rinaldi et al., 2007). Furthermore, a selective NMDA receptor antagonist with an Nr2b subunit specificity can attenuate the memory retention deficit in the water maze navigation caused by EtOH exposure in utero (Lewis et al., 2012), suggesting a true increase in Nr2b levels in some animal models of FASD. For the increased hippocampal Nr2b levels to have consequences in neuronal function and connectivity, the interaction between Nr2b and Rasgrf1 cannot be disrupted (Sepulveda et al., 2010). Thus, we believe that the opposite directional changes of Rasgrf1 and Nr2b levels in the EtOH hippocampus may lead to abnormal neuronal function and subsequently results in social behavior deficits.

It is of particular interest that the altered expression of all of these genes in the EtOH male hippocampus was reversed, or the difference reduced, by the low-dose T4 treatment, in parallel with the behavioral outcome. Additionally, the elevated Dio3 expression in the hippocampus of male offspring of EtOH-consuming dams was reversed by simultaneous administration of T4. The elevated hippocampal Dio3 expression in these offspring is in contrast to the decreased Dio3 expression described previously in the SD by Brown–Norway (BN) cross (Sittig et al., 2011b). The differences in these results are possibly due to strain differences, whereby the fetus that is exposed to EtOH is SD by BN hybrid in the previous study, but SD by SD in the present study.

The significance of prenatal T4-induced reversals in the altered expression of these genes in the EtOH male hippocampus needs to be determined. One appealing hypothesis is that prenatal EtOH-induced changes in thyroid function can alter expression of these ASD-related genes as most of them have thyroid responsive element (Table 5). Additionally, there are indications in the literature that some of these TREs are functional. In the rat hippocampus, mRNA levels of NMDA receptor subunits Nr1 and Nr2b are controlled by thyroid hormones (Lee et al., 2003). Binding and activity of the GABA-A receptor complexes of the brain are also modulated by thyroid hormones (Martin et al., 1996). Although we found no TREs in the promoter region of Dio3, its expression in the brain is known to be regulated by thyroid hormones (Tu et al., 1999).

It is important to note that Ube3a, Rasgrf1, and Dio3 are imprinted genes. UBE3A (and GABRB3) is mapped to human chromosome 15q11–13, a genetic locus implicated in autism (Cook et al., 1997). Imprinting of mouse Ube3a is thought to be region specific in the embryonic brain, and the sense transcript is expressed maternally in neurons (Yamashiki et al., 2003). As expected, the nonimprinted Gabrb3 shows biallelic expression; mononucleolar expression of Gabrb3 does not occur in normal brain (Hogart et al., 2007). Additionally, Rasgrf1 is an imprinted gene that is only expressed from the paternal allele in neonatal mouse brain until postnatal day 21, at which time expression becomes biallelic (Drake et al., 2009). Finally, we have shown previously that the preferential paternal expression of Dio3 in the frontal cortex of SD × BN cross is switched to preferentially maternal expression in the hippocampus (Sittig et al., 2011a,b). Similar to the changes in the allele-specific expression of Dio3, it is likely that maternal EtOH exposure affects not only the total, but also the allele-specific expression of 1 or more of these genes as well (Sittig et al., 2011b) and that such altered expression patterns contribute to the detrimental phenotypes associated with FASD.

**Thyroxin Supplementation and Reversal of Prenatal EtOH Effects**

It is well known that thyroid hormone is essential for normal brain development (Heindel and Zoeller, 2003). Even mild maternal hypothyroidism negatively affects a child’s neuropsychological development (Zoeller, 2004). It has been suggested that some of the prenatal EtOH-related deficits are due to the effects of EtOH on the disturbance of maternal-fetal thyroid homeostasis (Scott et al., 1998; Wilcoxon and Redei, 2004). Decreased levels of T4 have been found in alcoholics (Leggio et al., 2008), decreased serum TSH and T4 in alcohol-consuming pregnant women (Herbstman et al., 2007), and in newborns exposed to alcohol in utero (Hernandez et al., 1992). Similar findings in animal models are reported with decreased peripheral-free T4, fT3, and TSH in EtOH-consuming pregnant animals at gestational day 18 (Wilcoxon and Redei, 2004). In a more recent study, although plasma T4 and TSH levels are reported to be low, fT3 levels are found to be elevated in EtOH-consuming SD dams (Sittig and Redei, 2010). The thyroid function measures of the adult offspring of EtOH-consuming dams are similarly discordant. In the present study, adult male, but not female, offspring of EtOH-consuming dams showed decreased plasma TSH and increased fT3, which is a hyperthyroid profile. Prenatal EtOH-related changes in this hyperthyroid profile of plasma TSH/fT3 are the opposite of the hypothyroid profile of elevated TSH and decreased fT3 found previously in the offspring of EtOH-consuming dams (Wilcoxon and Redei, 2004), and the cause of this discrepancy is unknown. One potential explanation is within strain differences of SD rats used in the current study compared to those used previously. A similar difference in other measures has been noted previously (J. Weinberg, personal communication).

Regardless of differences in the thyroid function of both the EtOH-consuming dams and their adult offspring, administration of T4 reverses selected consequences of maternal EtOH exposure. Pharmacological dose of prenatal T4 treatment reverses the cognitive and motivational deficits (Wilcoxon et al., 2005), but suppresses plasma TSH and fT3 in the female EtOH offspring (Wilcoxon and Redei, 2004). The approximately 10 times lower dose of T4, administered in the current study, normalized the offspring’s thyroid function, eliminated their social interaction deficit and hippocampal expression differences induced by EtOH in the adult male offspring. These findings support the indispensable role of
appropriate maternal thyroid function in the normal neuro-psychological development of the fetuses, and manipulations of it could lead to potential treatment methods.

Using a rat model of FAE, we found that male, but not female, offspring of EtOH-consuming dams exhibited a significant impairment in both social interaction and memory. Significantly, these deficits were reversed by supplementation of the maternal EtOH diet with a low dose of T4 that has also restored thyroid function in the adult offspring. As several autism-related genes showed altered expression profiles in the male EtOH offspring that were normalized by T4 supplementation, ASD and FASD may share common affected molecular pathways.

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REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Adult female offspring of ethanol-consuming mothers (E) show no differences in peripheral thyroid measures from those of controls.

Fig. S2. Adult female offspring of dams on different diets exhibit significantly increased social interaction (A) and social memory (B).

Fig. S3. Relative quantification measured by real time RT-PCR indicates no differences in Gabrb3 (A), Ube3a (B), and Rasgrf1 (C) transcript levels in the frontal cortex of adult male offspring from different prenatal treatment groups.

Fig. S4. Protein levels of MeCP2 (A) and SLC25A12 (B) show no differences in the frontal cortex of adult male offspring from different prenatal treatment groups.