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ORIGINAL PAPER

Dihydromyricetin Prevents Fetal Alcohol Exposure-Induced Behavioral and Physiological Deficits: The Roles of $GABA_A$ Receptors in Adolescence

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Abstract Fetal alcohol exposure (FAE) can lead to a variety of behavioral and physiological disturbances later in life. Understanding how alcohol (ethanol, EtOH) affects fetal brain development is essential to guide the development of better therapeutics for FAE. One of EtOH's many pharmacological targets is the γ-aminobutyric acid type A receptor ($GABA_AR$), which plays a prominent role in early brain development. Acute EtOH potentiates inhibitory currents carried by certain $GABA_AR$ subtypes, whereas chronic EtOH leads to persistent alterations in $GABA_AR$ subunit composition, localization and function. We recently introduced a flavonoid compound, dihydromyricetin (DHM), which selectively antagonizes EtOH's intoxicating effects in vivo and in vitro at enhancing $GABA_AR$ function as a candidate for alcohol abuse pharmacotherapy. Here, we studied the effect of FAE on physiology, behavior and $GABA_AR$ function of early adolescent rats and tested the utility of DHM as a preventative treatment for FAE-induced disturbances. Gavage administration of EtOH (1.5, 2.5, or 5.0 g/kg) to rat dams on day 5, 8, 10, 12, and 15 of pregnancy dose-dependently reduced female/male offspring ratios (largely through decreased numbers of female offspring) and offspring body weights. FAE (2.5 g/kg) rats tested on postnatal days (P) 25–32 also exhibited increased anxiety and reduced pentylenetetrazol (PTZ)-induced seizure threshold. Patch-clamp recordings from dentate gyrus granule cells (DGCs) in hippocampal slices from FAE (2.5 g/kg) rats at P25-35 revealed reduced sensitivity of GABAergic miniature inhibitory postsynaptic currents (mIPSCs) and tonic current (I_{tonic}) to potentiation by zolpidem (0.3 μM). Interestingly, potentiation of mIPSCs by gaboxadol increased, while potentiation of I_{tonic} decreased in DGCs from FAE rats. Co-administration of EtOH (1.5 or 2.5 g/kg) with DHM (1.0 mg/kg) in pregnant dams prevented all of the behavioral, physiological, and pharmacological alterations observed in FAE offspring. DHM administration alone in pregnant rats had no adverse effect on litter size, progeny weight, anxiety level, PTZ seizure threshold, or DGC $GABA_AR$ function. Our results indicate that FAE induces long-lasting alterations in physiology, behavior, and hippocampal $GABA_AR$ function and that these deficits are prevented by DHM co-treatment of EtOH-exposed dams. The absence of adverse side effects and the ability of DHM to prevent FAE consequences suggest that DHM is an attractive candidate for development as a treatment for prevention of fetal alcohol spectrum disorders.

Keywords Fetal alcohol spectrum disorders · Pregnancy · Brain development · Anxiety · Seizure · Tolerance · Ethanol · Zolpidem · Gaboxadol · Dihydromyricetin

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Introduction

Fetal alcohol exposure (FAE) leads to a variety of behavioral disturbances later in life collectively referred to as fetal alcohol spectrum disorders (FASD) [1, 2]. The severity of the disturbances depends on pregnant mothers' alcohol (ethanol, EtOH) drinking patterns and doses. For example, approximately 5–20 % of pregnant women engage in moderate drinking (≥5 drinks/week) [3]. Many women keep drinking until they realize they are pregnant (4–6 weeks), and some of them quit or decrease their alcohol use only by mid-pregnancy [4–6], therefore many fetuses are exposed to EtOH during early stages of pregnancy. EtOH readily crosses the placenta; consequently, peak fetal blood EtOH levels are similar to the mother [7]. Although EtOH clearance is increased in pregnancy [8, 9], EtOH elimination capacity of the fetus is low, particularly in the early stages of pregnancy [10], and EtOH remains trapped in the amniotic fluid leading to reabsorption by the fetus, thereby prolonging exposure time [9, 11].

Neuro-pathophysiological mechanisms underlying EtOH's deleterious effects on brain development are poorly understood. One of EtOH's several major pharmacological targets is $GABA_A$Rs [12–14]. $GABA_A$Rs are heteropentameric ligand-gated ion channels formed from a family of 19 related subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3), some of which are more sensitive to acute potentiation by EtOH than others [15–17]. In the mature brain, chronic intermittent EtOH (CIE) administration leads to persistent alterations in $GABA_A$R subunit composition, localization, and function, reviewed in [13, 18]. $GABA_A$R plasticity is an important underlying mechanism of alcohol withdrawal symptoms, such as anxiety, hyperexcitability and tolerance to alcohol, as well as alcohol dependence [19–21]. By contrast, FAE effects on $GABA_A$Rs are not well understood, in part because $GABA_A$R subunit composition and function is highly developmentally regulated, with many subunit combinations expressed only postnatally [22, 23]. Better understanding of FAE-induced changes in brain development is essential to guide the development of better prevention and treatment strategies.

While numbers of women drinking alcohol during pregnancy appear to be decreasing in the US, as 12.2 % were reported in 1991–2005 [24] compared to 7.6 % in 2006–2010 [3], FASD collectively still represent the largest preventable set of birth defects [1, 2]. Furthermore, available medications for alcohol use disorders (acamprosate, naltrexone, and disulfiram) are not approved by the US Food and Drug Administration for use during pregnancy due to their multiple side effects, and no agents have yet progressed to the point of consideration for human trials [25].

We recently introduced a flavonoid compound, dihydromyricetin (DHM), as a candidate for alcohol abuse pharmacotherapy. We demonstrated that DHM is a weak positive modulator acting on $GABA_A$Rs, and that in vivo, DHM ameliorates acute alcohol intoxication and reduces alcohol withdrawal symptoms, while in vitro, DHM counteracts EtOH effects on $GABA_A$Rs and antagonizes EtOH exposure/withdrawal-induced $GABA_A$R plasticity [26]. In the present study, we test the hypothesis that FAE-induced neurobehavioral deficits involve alteration of $GABA_A$R function and that these deficits can be prevented by DHM administration.

Materials and Methods

Animals

All animal experiments followed The Institutional Animal Care and Use Committee approved protocols. The timed first pregnancy (~24 h accuracy) Sprague–Dawley young rats (~10–12 weeks old) were purchased and shipped from the supplier (Charles River Laboratories, Hollister CA) to the University of California, Los Angeles. Rats were housed in the vivarium under a 12 h light/dark cycle and had free access to food and water. Groups of pregnant dams received the following treatments: (1) EtOH (1.5 g/kg, gavage); (2) EtOH (2.5 g/kg); and (3) EtOH (5 g/kg); (4) EtOH (1.5 g/kg) + DHM (1 mg/kg); (5) EtOH (2.5 g/kg) + DHM (1 mg/kg); (6) EtOH (5.0 g/kg) + DHM (1 mg/kg); (7) DHM (1 mg/kg); (8) vehicle (water, 20 ml/kg). Dams were weighed and treated as above on the days 5, 8, 10, 12, and 15 of pregnancy but were otherwise undisturbed except for cage cleaning twice per week. Close to the parturition day, investigators took turn to continuously observe dams for signs of parturition, counted the number of newborn rats, and verified that no newborns were eaten. Offspring were counted on their birth date. On postnatal day 21 (P21), the offspring were weighed, separated by gender, and weaned. There were no offspring deaths between birth and weaning day in any of the experimental groups. Given the difficulty in determining the gender of offspring of dams treated with EtOH (5.0 g/kg), they were returned to their dams and weaned on P25 and their gender determined then. Subsequently, offspring were group-housed by gender. Behavioral and electrophysiological experiments were performed on P25-35 offspring.

Drugs

EtOH (Pharmco Products, Brookfield, CT) was administered by oral intubation (gavage) to dams at 1.5, 2.5 or 5 g/

kg of body weight as a 25 % (w/v) solution in drinking water. The control group received water (20 ml/kg of body weight). DHM (dihydromyricetin, (2R,3R)-3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-2,3-dihydrochromen-4-one. HPLC purified ≥98 %, Blue California, USA. Lot No. 2731-052708) was administered at 1 mg/kg in water by gavage.

Behavioral Assays

Offspring from treated dams were studied in behavioral assays on P25–32. The elevated plus maze assay (EPM) was performed in a quiet and dark room with only a low power red light. Rats were placed on the central area of the maze, and their behavior video recorded for 5 min. The number of entries into open arms, closed arms, or center platform and time spent in each of these areas were scored during off-line analysis. Data were reported as % of number of entries in arms, % of time spent in different arms, and number of total entries. For the pentylenetetrazol (PTZ) seizure assay, rats received an injection of PTZ (42 mg/kg, i.p.). The time to onset and the duration of tonic–clonic seizures were determined. For the loss of righting reflex (LORR) assay, rats were injected with EtOH (3 g/kg, i.p.) to induce LORR. After LORR onset, rats were placed on their backs in home cage and a timer started. The LORR period ends when the animal was able to flip over three times in 30 s.

Plasma EtOH Concentration Assay

Blood samples were collected from the tail vein of pregnant rats at different time points (30, 60, 120, and 180 min) after EtOH or EtOH + DHM gavage treatment as follows. The rat was briefly (<5 min) placed into a restraint tube; the tail vein at the tip of the tail was pricked with a sharp blade. Approximately 0.2 ml venous blood was placed into a capillary blood collection tube containing lithium heparin (Ram Scientific). Blood samples were centrifuged at 2,500 rpm for 20 min. The supernatant was collected and stored at −80 °C until assay. The EtOH content of each sample was measured in duplicate along with EtOH standards using the alcohol oxidase reaction procedure (GM7 Micro-Stat, Analox Instruments) [27].

Brain Slice Preparation and Recordings

Coronal slices (400 μm thick) of rat brain at the level of dorsal hippocampus were obtained using standard techniques [27]. Briefly, P25–35 rats were decapitated under isoflurane anesthesia, brains were quickly removed, trimmed with a razor blade and glued to the base of a cutting chamber (Leica VT1200S) filled with cold (~4 °C) artificial cerebrospinal fluid (ACSF) composed of 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (Sigma-Aldrich Co, MO). The ACSF was continuously bubbled with a 95/5 % mixture of O_2/CO_2 to ensure adequate oxygenation of slices and a pH of 7.4. Patch electrode filling solutions contained: 135 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 mM HEPES, 2 mM K_2ATP, 0.2 mM Na_2GTP; pH adjusted to 7.25 with CsOH. Whole-cell patch clamp recordings were obtained from dentate granule cells (DGCs) during perfusion with oxygenated ACSF at 34 ± 0.5 °C. DGCs were voltage-clamped at −70 mV (CsCl) with the aid of a Multiclamp 700B amplifier and a Digidata 1440 A/D converter (Molecular Devices, Sunnyvale, CA). $GABA_A$R-mediated currents were separated pharmacologically by application of TTX (0.5 μM), CNQX (10 μM), APV (40 μM), and CGP54626 (1 μM) in the ACSF. Picrotoxin, zolpidem and gaboxadol (4,5,6,7 tetrahydroisoxazolo [5,4-c] pyridin-3-ol, THIP) (all from Sigma-Aldrich) were applied after appropriate dilution in the ACSF. The recordings were low-pass filtered (Clampfit software, Molecular Devices, Sunnyvale, CA) at 2 kHz and analyzed with the aid of the Mini Analysis Program (Synaptosoft Inc., Fort Lee, NJ). The mIPSCs were detected with threshold criteria of: 5 pA, amplitude and 20 fC, charge transfer. Frequency of mIPSCs was determined from all automatically detected events in a given recording period. The tonic current magnitudes were obtained from the mean baseline current of a given recording period. For kinetic analysis of mIPSCs, single events with a stable baseline, sharp rising phase and exponential decay were chosen. Double and multiple peak mIPSCs were excluded. The mIPSC kinetics were obtained from analysis of the averaged chosen single events (>120 events/100 s recording period) aligned with half rise-time in each cell.

Statistics

The investigators performing the behavioral tests, electrophysiological recordings and analysis were blind to treatments (vehicle, EtOH, DHM, or EtOH + DHM) received by the dams. All summary values are presented as mean ± SEM. Group differences were evaluated by one-way or two-way analysis of variance (ANOVA) or repeated measures (RM) ANOVA, followed by a post hoc multiple comparison based on Holm-Sidak method when ANOVA showed statistical significance. $p < 0.05$ was considered statistically significant.

Results

Fetal Alcohol Exposure Induces Deficits in Growth and Gender-Specific Birth Rates

We first assessed the dose-dependent effects of fetal EtOH exposure on the numbers, gender, and body weights (BW) of offspring from dams treated with vehicle (water) or EtOH (1.5, 2.5, or 5.0 g/kg) on day 5, 8, 10, 12, and 15 of pregnancy (Fig. 1a).

Pregnancy lasted 21–22 days. There was a trend to decreased body weight of dams treated with various EtOH doses which reached statistical significance for dams treated with 1.5 or 2.5 g/kg of EtOH by day 15 of pregnancy (Table 1). Dams built nests prior to parturition. Stretching and hindleg extension were usually signs of impending birth. Parturition occurred at night with 8–15 pups being born over a course of 1–4 h. Pups were born either head or tail first (breech). The dams ate the placenta. Offspring were counted on their birth date whereas their body weights and gender were determined on weaning day (P21) in order to minimize interference with dams and their newborns. Compared to offspring of vehicle-treated dams (14.5 ± 0.24), the number of offspring from each of the three groups of EtOH-treated dams (1.5 g/kg: 12.6 ± 0.34; 2.5 g/kg: 12.3 ± 0.29; 5 g/kg: 9.3 ± 0.88) was significantly decreased ($p < 0.001$, one-way ANOVA). All offspring survived to their weaning day. The number of male offspring from the 2.5 and 5.0 g/kg EtOH dams slightly increased, while the female offspring numbers significantly decreased in all EtOH groups (Fig. 1b). Surprisingly, two of three dams treated with 5 g/kg EtOH delivered male offspring only. The results indicate that FAE not only reduces litter size, but also affects offspring gender ratios (male vs., female: vehicle: 1.02; EtOH (1.5 g/kg): 2:1; EtOH (2.5 g/kg): 3:1; EtOH (5 g/kg): 13:1). Both male and female offspring of EtOH-treated dams exhibited lower body weights compared to the appropriate gender offspring of vehicle-treated dams (Fig. 1c). The decreases in offspring body weight were a function of increasing EtOH concentrations; the greatest decreases were observed in offspring of dams treated with 5 g/kg of EtOH (Fig. 1c).

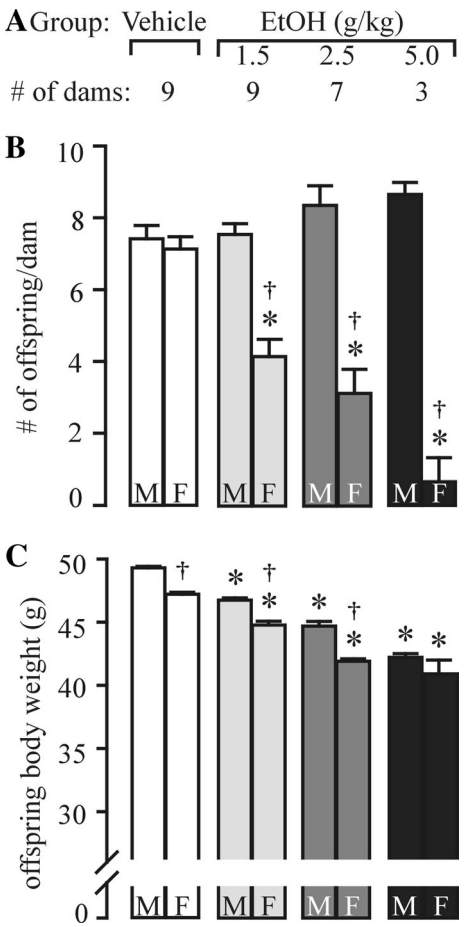

Fig. 1 Fetal EtOH exposure dose-dependently induces growth deficits and decreases numbers of female offspring. **a** Pregnant dam treatment groups and dam numbers. **b** Average male and female offspring numbers per dam. A two-way repeated measures (RM) ANOVA with repeated measures on one factor, "gender", was performed. The interaction between gender and treatment was highly significant ($p < 0.01$). Therefore, post hoc multiple comparisons with Holm-Sidak method was performed. **c** Average male and female offspring body weights at weaning (P21). A two-way ANOVA followed by a post hoc multiple comparison with Holm-Sidak method was performed. For **b** and **c**: $^{*}p < 0.05$ treatment versus the vehicle group of the corresponding gender. $^{\dagger}p < 0.05$, female versus male for each specific treatment

DHM Co-administration Prevents FAE-Induced Birth and Growth Deficits

In previous studies, we showed that dihydromyricetin (DHM) could counteract the effects of EtOH intoxication in adult rats [26]. Here we tested whether DHM could prevent the detrimental consequences of fetal alcohol exposure on offspring. We co-administered EtOH at 1.5 or 2.5 g/kg with DHM 1 mg/kg and DHM alone to dams on day 5, 8, 10, 12, and 15 of pregnancy (Fig. 2a). The offspring body weight from the DHM alone or the EtOH (1.5 and 2.5 g/kg) plus DHM groups did not differ from that of the vehicle group (Fig. 2b). Furthermore, the number of offspring of dams treated with DHM alone or with EtOH + DHM, as well as their male: female ratios did not differ from those of the vehicle group (Fig. 2c). These results indicate that DHM administered alone has no detrimental effects on offspring number, body weights, or gender ratios and that when co-administered with EtOH, DHM prevents FAE-induced birth and growth deficits.

Table 1 Dam body weights on drug administration days of pregnancy (E)

Group	Dam body weight (g)					
	E5	E8	E10	E12	E15	n
Vehicle	235.3 ± 2.5	241.0 ± 3.1	249.5 ± 3.4	260.0 ± 3.5	271.0 ± 4.1	9
EtOH (1.5 g/kg)	234.6 ± 2.6	240.8 ± 3.5	248.5 ± 3.0	259.0 ± 3.5	267.5 ± 3.6	9
EtOH (2.5 g/kg)	235.5 ± 3.1	240.0 ± 3.2	245.5 ± 3.0	252.0 ± 2.9	261.0 ± 3.5*	7
EtOH (5.0 g/kg)	240.0 ± 2.5	246.1 ± 2.2	253.3 ± 3.5	257.5 ± 3.5	262.4 ± 3.7*	3
EtOH (1.5 g/kg) + DHM (1 mg/kg)	235.3 ± 2.8	240.9 ± 3.2	249.7 ± 3.3	260.2 ± 3.1	270.9 ± 3.6	8
EtOH (2.5 g/kg) + DHM (1 mg/kg)	233.7 ± 2.5	240.2 ± 3.0	247.5 ± 3.5	256.2 ± 2.5	267.8 ± 3.1	7
DHM (1 mg/kg)	240.0 ± 1.5	246.2 ± 2.5	255.2 ± 3.1	261.5 ± 3.5	272.4 ± 3.8	6

* $p < 0.05$ versus Vehicle group (two-way RM ANOVA) followed by a post hoc multiple comparison with Holm-Sidak method

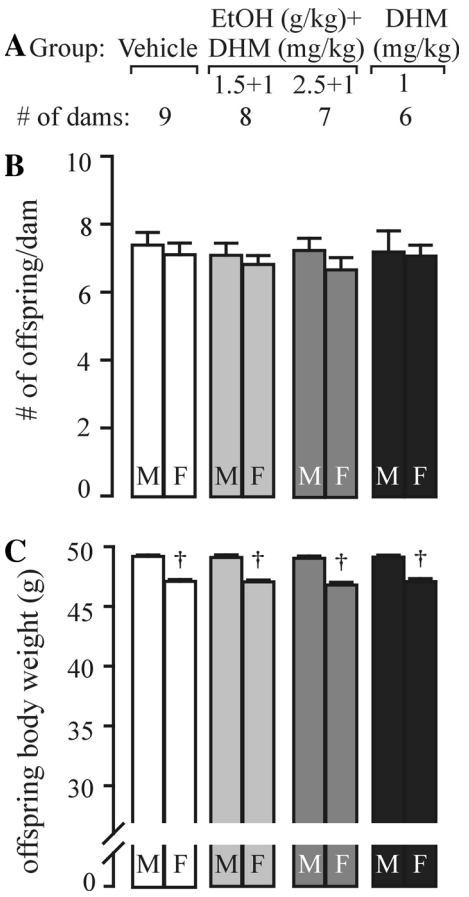

Fig. 2 DHM co-administration with EtOH in dams prevents EtOH-induced decreases in offspring numbers and weights. **a** Pregnant dam treatment groups and dam numbers. **b** Average male and female offspring numbers per dam. The same two-way RM ANOVA was performed as described in Fig. 1b legend. No significant differences were found. **c** Average male and female offspring body weights. The same two-way ANOVA was performed as described in Fig. 1c legend. No significant differences were found

DHM Co-administration Decreases Blood EtOH Levels in Pregnant Dams

Previous studies demonstrated that DHM co-administration decreased plasma EtOH concentrations in adult male rats [26]. Since the reduction in plasma EtOH concentrations could contribute to the mechanisms of DHM's prevention of FAE-induced birth and growth deficits, we tested if DHM would similarly affect plasma EtOH levels of pregnant dams. We took venous blood samples at 3, 15, 60, 120, and 180 min from dams treated with EtOH alone and EtOH plus DHM on their last treatment day (15th day of pregnancy) and processed them for plasma [EtOH] measurements. After EtOH administration (2.5 and 5 g/kg, gavage), plasma [EtOH] increased slowly to peak at 60 min, followed by a very slow gradual decline (Fig. 3). The peak plasma [EtOH] of ~160 mg/dl after the 2.5 g/kg dose is similar to that achieved in humans after a moderate level of social drinking. Co-administration of EtOH (2.5 g/kg) with DHM (1 mg/kg), resulted in significant decreases of plasma [EtOH] at the 120, and 180 min time points compared to the EtOH alone group. These results suggest that DHM co-administration decreases blood EtOH levels in pregnant dams and that this can contribute to the protective effects of DHM against FAE-induced abnormalities.

DHM Co-administration Prevents FAE-Induced Behavioral Alterations

$GABA_AR$ functional alterations are well-known to affect anxiety behaviors [28–30], seizure susceptibility [31–33], and sensitivity to anesthetic/soporific effects of acute EtOH administration in the adult brain [13, 18]. To determine the effect of FAE on these parameters later in life we first assessed offspring anxiety levels in the elevated plus maze (EPM). For each experimental group there were 2–4 males and 1–3 females chosen from the same litter. Both male and female offspring of the EtOH (2.5 g/kg) group tested at P25–32 spent significantly less time in the open arms and more time in closed arms, an indication of increased anxiety levels, compared to the offspring from the vehicle-treated group (Fig. 4a). By contrast, the offspring of the DHM only and the EtOH + DHM-treated dams spent similar time in the open and closed arms as the offspring of the vehicle-treated group (Fig. 4a).

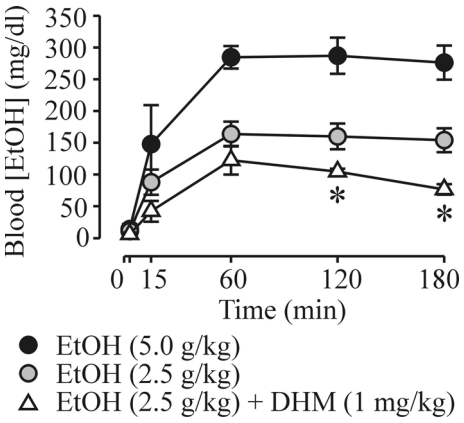

Fig. 3 Co-administration of DHM with EtOH decreases plasma [EtOH]. Dams were treated with EtOH (2.5 or 5 g/kg, gavage) or EtOH (2.5 g/kg) with DHM (1 mg/kg). Blood samples were collected at 3, 15, 60, 120 and 180 min after EtOH administration. Two-way RM ANOVA revealed highly significant ($p < 0.01$) interaction of time by treatment. Therefore, post hoc multiple comparisons with Holm-Sidak method followed. The value at every time point of every treatment group versus the corresponding pre-treatment (time 0) value was significant (for simplicity, we did not put *symbols* to show this). *$p < 0.05$ EtOH (2.5 g/kg) + DHM group versus EtOH (2.5 g/kg) group (n = 3–4 dams per group) at the time point

The sensitivity to the acute anesthetic/soporific effects of EtOH (3 g/kg, i.p.) was addressed using the loss of righting reflex (LORR) assay in male and female offspring previously studied in the EPM. There was a significant difference in mean LORR duration between male and female FAE offspring (Fig. 4b), consistent with the known gender differences in sensitivity to EtOH [34, 35]. Compared with the LORR duration in the vehicle group, FAE offspring of both genders tended to have a reduced duration of LORR (Fig. 4b. Not statistically significant; $p = 0.25$). By contrast, the offspring of the DHM only and the EtOH + DHM-treated dams did not show this trend (Fig. 4b).

Hyperexcitability was assessed with the PTZ-induced seizure assay in P27–34 offspring 2 days after the LORR assays. Overall, female rats tended to have longer time to onset (Fig. 4c) and shorter duration (Fig. 4d) of PTZ-induced seizures (not statistically significant), consistent with known gender differences in PTZ sensitivity [36]. We also observed a trend of shorter seizure onset time after PTZ injection in the EtOH group offspring compared to the offspring from the vehicle-treated groups of both genders which did not reach statistical significance (Fig. 4c). By contrast, the offspring of the DHM alone and the EtOH + DHM groups showed seizure onset times similar to the offspring of the vehicle-treated group (Fig. 4c). PTZ-induced seizure duration was significantly longer in the offspring of EtOH-treated dams compared to that of the vehicle group offspring (Fig. 4d). By contrast, seizure duration in the offspring of DHM only and the EtOH + DHM-treated dams was similar to the offspring of the vehicle-treated group (Fig. 4d). These results suggest that FAE induces long-lasting increases in postnatal anxiety, tolerance to acute sedative/anesthetic effects of EtOH, and increases seizure susceptibility. All of these FAE-induced changes were prevented by DHM co-administration in dams.

DHM Prevents FAE-Induced Changes in Postsynaptic $GABA_AR$ Function

The observed behavioral and pharmacological manifestations of FAE were suggestive of altered GABAergic inhibition. The plasticity of $GABA_AR$ in hippocampal neurons induced by acute and chronic EtOH is likely to be representative of the same sort of EtOH-sensitive $GABA_AR$ in many brain regions including those cells responsible for the behavioral observations. Therefore, we assessed the kinetics and pharmacological sensitivity of $GABA_AR$-mediated miniature postsynaptic inhibitory currents (mIPSCs) and tonic inhibitory current (I_{tonic}) in dentate gyrus granule cells (DGCs) in hippocampal slices from offspring (P25–35) of dams exposed to vehicle, EtOH (2.5 g/kg), DHM (1 mg/kg), or EtOH + DHM treatments. All recordings were performed in hippocampal slices from offspring that were not previously subjected to behavioral and pharmacological tests. First we compared the basal magnitude of the picrotoxin-sensitive I_{tonic} and mIPSC kinetics for differences between genders and different treatment groups (Table 2). In the vehicle-treated groups, the only difference between genders was a smaller mIPSC charge transfer in the female progeny. In the FAE groups, there was a trend to decreased magnitude of I_{tonic} in both male and female FAE progeny, but it did not reach statistical significance. The frequency of mIPSCs was not altered in offspring from EtOH-treated dams or other treatment groups (Table 2). Similarly, the rise-time, amplitude, and decay time constant ($\tau 1$) were not affected by EtOH, DHM, or EtOH + DHM treatments. However, the decay $\tau 2$ of mIPSCs from FAE rats of both genders was significantly increased, suggestive of changes in postsynaptic GABAergic function. The total charge transfer of mIPSCs was also significantly larger in FAE rats. All of the FAE-induced changes in $GABA_AR$ currents were prevented by DHM co-administration in dams, while DHM treatment alone did not alter any of the recorded parameters (Table 2).

DHM Prevents FAE-Induced Decreases in Zolpidem Potentiation of Hippocampal $GABA_ARs$

Postsynaptic function and pharmacological sensitivity of $GABA_ARs$ is largely dictated by their subunit composition

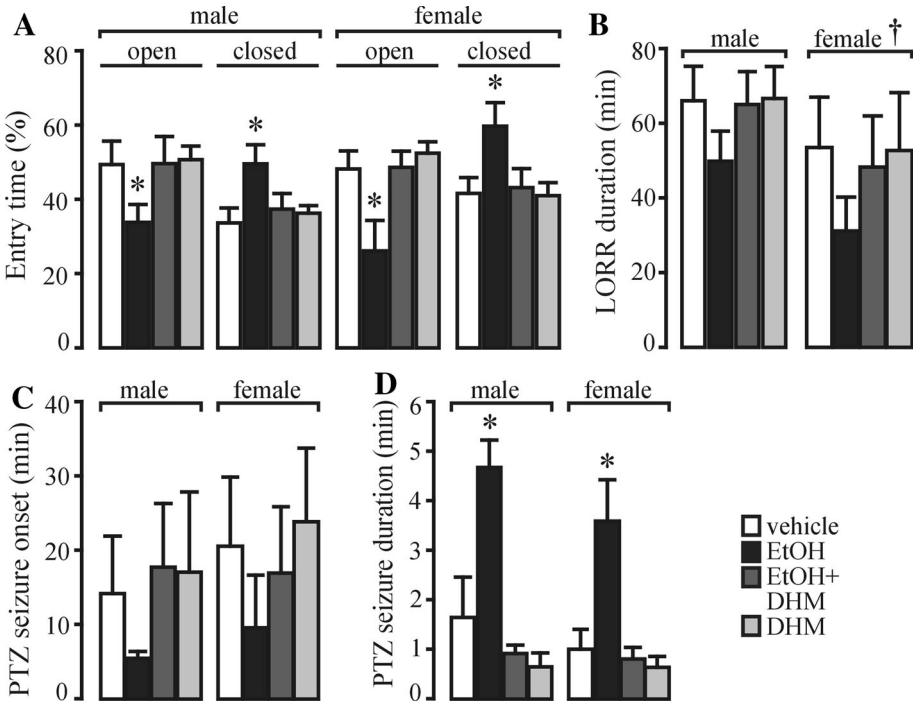

Fig. 4 Co-administration of DHM with EtOH in pregnant dams prevents FAE-induced behavioral and physiological abnormalities in their offspring. **a** Anxiety in male and female offspring was measured by elevated plus maze (n = 8 offspring per group). Both male and female offspring of EtOH (2.5 g/kg)-treated pregnant dams spent less time in the open arms and more time in the closed arms compared to the vehicle group. The EtOH + DHM group spent a similar amount of time in each arm as the vehicle group. Two-way ANOVA for open and separately for closed arm entry durations followed by post hoc multiple comparisons based on Holm-Sidak method were performed. *$p < 0.05$ treatment versus vehicle group. **b** Sensitivity to anesthetic effect of EtOH (3 g/kg, i.p.) was measured by LORR in rats from **a**. There was a significant difference in mean LORR duration between male and female FAE offspring (overall gender difference $p = 0.047$). The EtOH groups of both genders showed a trend to shorter duration of acute EtOH-induced LORR which did not reach statistical significance (overall group effects: $p = 0.25$). The DHM alone and EtOH + DHM groups did not show this trend. **c** At 2 days after LORR testing, the offspring of EtOH-treated dams showed a trend to shorter time to onset of PTZ-induced seizures which did not reach statistical significance (overall group effects: $p = 0.627$). The DHM and EtOH + DHM groups showed similar seizure onset time as the vehicle groups. **d** The duration of PTZ-induced seizures was significantly longer in the offspring of EtOH-treated dams. The DHM alone and EtOH + DHM groups showed similar duration of seizures as the vehicle group. For **b–d**, two-way ANOVA followed by post hoc multiple comparisons based on Holm-Sidak were performed. *$p < 0.05$ treatment versus the vehicle control group. $^{\dagger}p < 0.05$ females versus males

Table 2 Tonic current magnitude and kinetic properties of mIPSCs from offspring DGC recordings

Gender	Group	I_{tonic} (pA)	Frequency (Hz)	Rise-time (ms)	Amplitude (pA)	Decay τ1 (ms)	Decay τ2 (ms)	Area (fC)	Cells/ progeny
Male	Vehicle	27.8 ± 1.5	13.3 ± 0.1	0.98 ± 0.05	33.6 ± 3.5	6.7 ± 0.5	27.7 ± 4.0	465.9 ± 21.3	11/3
	EtOH (2.5 g/kg)	22.5 ± 1.3*	13.0 ± 0.2	1.02 ± 0.03	33.8 ± 2.2	7.3 ± 0.4	48.3 ± 6.1*	549.2 ± 19.6*	18/4
	EtOH (2.5 g/kg) +DHM (1 mg/kg)	26.0 ± 1.3	13.3 ± 0.1	0.97 ± 0.05	33.9 ± 3.1	6.7 ± 0.3	27.2 ± 3.6	469.4 ± 18.3	19/4
	DHM (1 mg/kg)	28.1 ± 1.7	13.3 ± 0.1	0.99 ± 0.05	33.7 ± 2.6	6.7 ± 0.3	30.6 ± 6.3	465.18 ± 25.0	10/3
Female	Vehicle	26.7 ± 1.5	13.4 ± 0.1	0.98 ± 0.11	32.0 ± 0.5	6.6 ± 0.4	21.3 ± 3.2	398.0 ± 21.3†	8/2
	EtOH (2.5 g/kg)	21.1 ± 1.4*	13.0 ± 0.1	1.01 ± 0.03	34.3 ± 3.4	6.5 ± 0.3	41.4 ± 7.4*	503.1 ± 20.4*	15/3
	EtOH (2.5 g/kg) +DHM (1 mg/kg)	26.2 ± 1.5	13.3 ± 0.1	1.01 ± 0.03	32.8 ± 2.2	6.6 ± 0.3	24.3 ± 2.0	438.9 ± 21.3	25/5
	DHM (1 mg/kg)	27.5 ± 1.7	13.3 ± 0.1	0.99 ± 0.03	33.2 ± 2.3	6.4 ± 0.4	26.3 ± 3.3	448.4 ± 25.0	9/2

* $p < 0.05$, treatment group versus vehicle group

† $p < 0.05$, female versus male with the same treatment (two-way ANOVA followed by a post hoc multiple comparison with Holm-Sidak method)

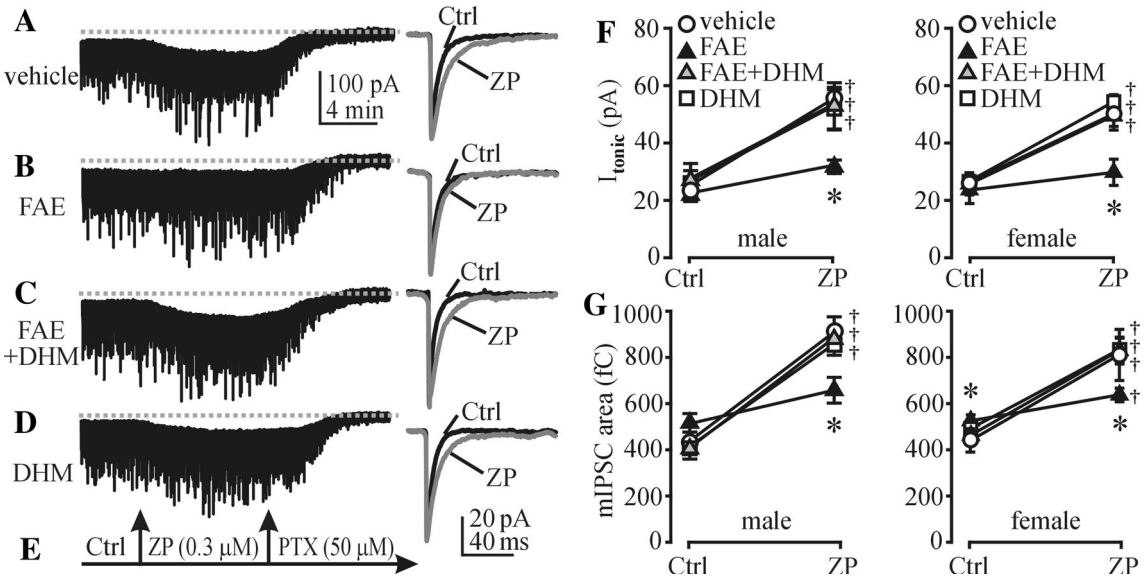

Fig. 5 Co-administration of DHM with EtOH in pregnant dams prevents FAE-induced decreases in potentiation of synaptic and extrasynaptic $GABA_A$Rs by zolpidem. **a–d** Recordings (*left traces*) of pharmacologically-isolated $GABA_A$R currents in dentate granule cells from male offspring of differentially-treated dams. *Gray dashed lines* show the picrotoxin (PTX, 50 μM)-sensitive tonic current (I_{tonic}). Superimposed mIPSCs (*right traces*) are averaged from the 100 s recording periods before (Ctrl) and during zolpidem (ZP, 0.3 μM) application. **e** The recording protocol. **f** Summary of group differences in I_{tonic} sensitivity to ZP for male (*left graph*) and female (*right graph*) progeny. **g** Summary of group differences in mIPSC sensitivity to ZP for male (*left graph*) and female (*right graph*) progeny. $^{\dagger}p < 0.05$, versus Ctrl; $^{*}p < 0.05$, versus offspring of vehicle-treated dams (n = 4–6 cells from 2 to 3 male offspring per group; n = 4 cells from 1 to 2 female offspring per group), two-way RM ANOVA followed by a post hoc multiple comparison with Holm-Sidak method

[37] and subject to some fine tuning by phosphorylation/dephosphorylation states, e.g., [38]. Therefore, we tested $GABA_A$R-currents for sensitivity to zolpidem (ZP), a benzodiazepine site positive modulator of $GABA_A$R function with selectivity for the α1-containing $GABA_A$Rs, intermediate potency at α2/3-containing $GABA_A$Rs, and inactivity at α4/5/6-containing $GABA_A$Rs [39]. In DGCs from vehicle-treated dams bath application of ZP potentiated both I_{tonic} magnitude (Fig. 5a, f) and mIPSC charge transfer (Fig. 5a, g). The ZP responsiveness of both I_{tonic} and mIPSCs was significantly reduced in recordings from DGCs of FAE offspring compared to offspring of vehicle-treated dams (Fig. 5b, g, f). These decreases in ZP responsiveness were observed in recordings from DGCs of both male and female offspring (Fig. 5f, g). By contrast, ZP responsiveness of I_{tonic} and mIPSCs from DHM alone and EtOH + DHM groups was indistinguishable from that of the vehicle group offspring (Fig. 5a, c, d, g, f). These results indicate that FAE alters postsynaptic $GABA_A$R function (Table 2) and ZP sensitivity (Fig. 5) and that these alterations are prevented by co-administration of DHM in dams. These results further suggest that the FAE-induced decreases in ZP sensitivity are likely due to decreased function and/or expression of α1 subunit-containing synaptic and/or extrasynaptic $GABA_A$Rs.

DHM Prevents FAE-Induced Changes in Responsiveness of Synaptic and Extrasynaptic $GABA_A$Rs to THIP

Previous studies have demonstrated that chronic EtOH exposure in adult rodents leads not only to decreases in α1 and δ subunit-containing $GABA_A$Rs, but also to increased expression and altered localization of α4-containing $GABA_A$Rs [13, 21]. To determine if similar alterations in expression and localization of α4-containing $GABA_A$Rs occur after FAE we tested $GABA_A$R-currents for sensitivity to gaboxadol (4,5,6,7 tetrahydroisoxazolo [5,4-*c*] pyridin-3-ol, THIP), which is a selective $GABA_A$R agonist with a preference for α4/δ-containing extrasynaptic $GABA_A$Rs [40–45]. In DGCs from offspring of vehicle-treated dams, bath application of THIP (0.1 μM) potentiated both I_{tonic} magnitude (Fig. 6a, f) and mIPSC charge transfer (Fig. 6a, g). The potentiation of I_{tonic} by THIP was significantly reduced in DGCs of FAE offspring compared to offspring of vehicle-treated dams (Fig. 5b, f, g). By contrast, THIP potentiation of mIPSCs was increased in offspring of EtOH-treated dams. These alterations in THIP responsiveness of synaptic and extrasynaptic $GABA_A$R currents were observed in DGCs of both male and female offspring (Fig. 6f, g). In recordings from the offspring of

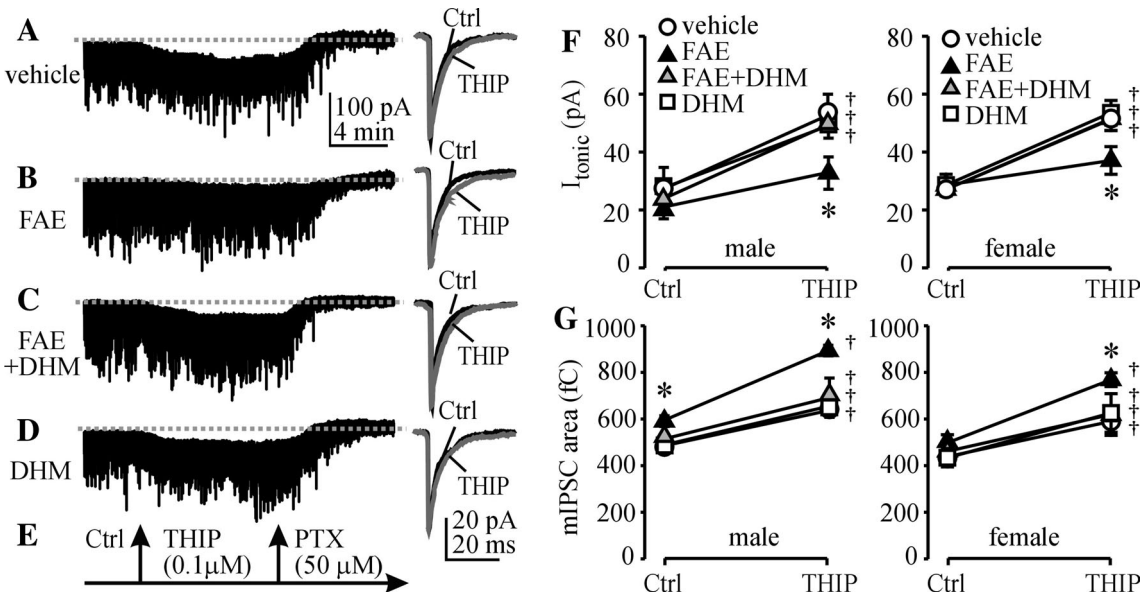

Fig. 6 Co-administration of DHM with EtOH in pregnant dams prevents FAE-induced changes in sensitivity of synaptic and extrasynaptic $GABA_A$Rs to THIP. **a–d** Recordings (*left traces*) of pharmacologically-isolated $GABA_A$R currents in dentate granule cells from male offspring of differentially-treated dams. *Gray dashed lines* show the picrotoxin (PTX, 50 μM)-sensitive tonic current (I_{tonic}). Superimposed mIPSCs (*right traces*) are averaged from the 100 s recording periods before (Ctrl) and during THIP (0.3 μM) application. **e** The recording protocol. **f** Summary of group differences in I_{tonic} sensitivity to THIP. **g** Summary of group differences in mIPSC area sensitivity to THIP. $^{\dagger}p < 0.05$ versus Ctrl; $^{*}p < 0.05$ versus offspring of vehicle-treated dams (n = 4 cells from two male offspring per group; n = 4 cells from two female offspring per group), two-way RM ANOVA followed by a post hoc multiple comparison with Holm-Sidak method

the DHM alone and EtOH + DHM groups, THIP responsiveness of I_{tonic} and mIPSCs was indistinguishable from that of the vehicle group offspring (Fig. 6a, c, d, f, g). These results indicate that the FAE-induced alterations in THIP sensitivity are prevented by co-administration of DHM. These results also suggest that FAE-induced differential changes in THIP sensitivity of I_{tonic} and mIPSCs are likely due to alterations in expression and/or localization of α4/δ-containing $GABA_A$Rs at extrasynaptic and synaptic locations.

Discussion

FAE and DHM Effects on Birth and Growth Deficits

We show that intermittent EtOH (1.5, 2.5, or 5.0 g/kg) administration on day 5, 8, 10, 12, and 15 of pregnancy in rats leads to dose-dependent decreases in litter sizes and weights at weaning. This is consistent with numerous reports of FAE-induced decreases in rat offspring numbers and birth weights [46–51]. In humans, FAE is also correlated with low birth weight, growth, and morphological abnormalities [4], and in higher rates of spontaneous abortions [52, 53]. Various potential mechanisms have been proposed to account for the birth and growth deficits of FAE rats including, but not limited to, interference with chromosome segregation [54], epigenetic reprogramming [55–57], deficient choline metabolism [58], blunting of cellular immunity [59], oxidative stress [60], as well as decreased production and release of various growth factors and hormones [61–64]. We also observed that the FAE-induced decreases in litter sizes were primarily due to decreased numbers of female offspring. Since gender is determined at conception and embryos were first exposed to EtOH only 5 days later, the lower female/male ratios may have resulted from preferential decreases in the in utero viability of female offspring. While we have not found references to FAE-induced changes in gender ratios in the literature, previous studies have demonstrated an in utero "feminization" of FAE male rodents and "masculinization" of female rodents, attributable to EtOH-induced derangements of in utero androgenesis [65, 66]. Studies have also shown that maternal exposure to EtOH during the critical period of rat organogenesis preferentially affects body weights and increases malformations in offspring dependent on their in utero contiguity to siblings of the opposite sex [67]. Thus, the observed decreases in male offspring body weights could have been exaggerated due to reduced in utero influence of viable female siblings.

We showed that the FAE-induced birth and growth deficits were completely prevented by co-administration of

DHM with EtOH to pregnant rats. We also showed that DHM (1 mg/kg) co-administration with EtOH (2.5 g/kg) decreases plasma [EtOH] in dams, consistent with similar findings in adult male rats [26]. Some flavonoids do affect ethanol metabolism, probably peripherally. It has been reported that *Hovenia,* the herb from which we originally extracted DHM, promotes alcohol clearance, and enhances the activity of alcohol metabolic enzymes, alcohol dehydrogenase and aldehyde dehydrogenase [68, 69], thereby decreasing blood EtOH concentrations. We think that the reversal of birth and growth deficits by DHM is more likely pharmacodynamic than pharmacokinetic. Previous studies have demonstrated that administering EtOH at doses as low as 1 g/kg to pregnant rat dams produces behavioral and physiological abnormalities in their offspring, including higher neonatal mortality and lower birth weights [48]. In our study, DHM reduced plasma [EtOH] to levels that might be achieved after administration of a lower (1.5–2 g/kg) EtOH dose [70–72]. However, we also showed that birth and growth deficits were still evident in the offspring of the 1.5 g/kg EtOH alone group without DHM (Fig. 1), whereas such deficits were prevented after DHM co-treatment of the 2.5 g/kg EtOH group (Fig. 2). This suggests that the complete prevention of FAE-induced decreases in litter sizes and weights cannot be attributed solely to DHM-induced decreases in plasma [EtOH]. One plausible mechanism is the ability of DHM to block EtOH actions at $GABA_A$Rs [26].

GABA and $GABA_A$Rs are essential regulators of early brain development and disruption of GABAergic signaling is suspected in numerous neurodevelopmental disorders [73–78]. We observed increased anxiety in the EPM and faster onset, as well as longer duration of PTZ-induced seizures after FAE in P25-34 rats (Fig. 4), which corresponds to the beginning ($\sim$P28) of the adolescent period in rats [79]. To our knowledge, this is the first demonstration of heightened anxiety in FAE rats during early adolescence. Previous studies have demonstrated increased anxiety and depressive behaviors in adult FAE rats [60, 80–82], however see [83]. Children diagnosed with FASD exhibit high anxiety [84, 85], as well as a remarkably high prevalence of epilepsy [86]. While other mechanisms may also be involved, decrements in GABAergic neurotransmission have been associated with increased anxiety [29, 30, 81] and reduced seizure thresholds [33, 87].

Cognitive impairment is also prominent in children diagnosed with FASD [1, 2]. Numerous studies have demonstrated impairment in various learning and memory tasks in adolescent and adult FAE rats, including hippocampal-mediated spatial reference memory [88–93]. Both DGCs and hilar GABAergic interneurons of the dentate gyrus were demonstrated to be integral neural substrates for spatial reference and spatial working memory [94–97]. Interestingly, THIP-sensitive $GABA_A$Rs containing the δ subunit promote memory and neurogenesis in the dentate gyrus [98]. We observed a trend to decreased magnitude of I_{tonic} and significant increases in charge transfer (and decay τ2) of mIPSCs in DGCs of both male and female FAE progeny (Table 2). We also observed altered pharmacological responsiveness of DGC $GABA_A$Rs from FAE rats to zolpidem and THIP (Figs. 5, 6). It is therefore likely that the FAE-induced alterations in hippocampal $GABA_A$Rs contribute to the deficits in hippocampal learning and memory in FAE rats.

Prior studies also have shown that the responsiveness of GABA-stimulated $^{36}Cl^-$ flux to benzodiazepine site ligands and to neurosteroids is altered in a brain region-specific manner in FAE rats [99]. Studies have also demonstrated FAE-induced decreases in allopregnanolone potentiation of [^{3}H]flunitrazepam binding to hippocampal $GABA_A$Rs in guinea pigs [100], age-dependent changes in α5 $GABA_A$R subunit expression in mice [101], and increased δ $GABA_A$R subunit expression and sensitivity to THIP in cerebellar granule neurons of rats after early postnatal (P2–12) EtOH vapor exposure [102]. Neurosteroids, such as 3α,5α-3-hydroxypregnan-20-one (3α,5α-THP or allopregnanolone) and 3α;5α-tetrahydrodeoxycorticosterone (THDOC), are potent endogenous modulators of δ subunit-containing $GABA_A$Rs [103]. Acute EtOH dynamically regulates local 3α,5α-THP levels in several subcortical regions of adult rats, independent of the adrenal glands [104, 105]. Several lines of evidence also suggest that 3α,5α-THP and THDOC contribute to EtOH's pharmacological effects in rats [106]. The levels of these and other neurosteroids change dramatically during embryonic development, parturition, and postnatal development [107–109]. Other studies showed a direct link between neurosteroid concentrations and alterations in $GABA_A$R expression and function [110–114]. Importantly, FAE has been shown to affect neurosteroid synthesis [115] and to decrease both cellular and behavioral responsiveness to neurosteroids [116, 117]. Collectively these studies demonstrate that fetal and early postnatal EtOH exposure leads to brain region-selective alterations in subunit composition and function of $GABA_A$Rs and that while alterations in neurosteroidogenesis likely play a role, the precise molecular and cellular mechanisms by which FAE exposure alters $GABA_A$R function remain to be determined.

The decreases in zolpidem sensitivity of adult CNS neurons have been related to chronic EtOH-induced impairment of α1-containing $GABA_A$Rs, usually as a result of decreases in membrane surface (functional) expression of α1-containing $GABA_A$Rs [13, 18]. By contrast, the EtOH-induced switch in the relative responsiveness of synaptic and tonic $GABA_A$R currents to THIP has been previously related to decreases in membrane surface

expression of extrasynaptic α4βδ $GABA_A$Rs and compensatory increases in α4βγ2 $GABA_A$Rs at synaptic and extrasynaptic locations [27, 42, 118, 119]. The transcriptionally-mediated increases in α4 were shown to be due to EtOH-induced activation of heat shock factor 1 and its binding to a short downstream DNA sequence, the alcohol response element on the Gabra4 gene [120]. Protein kinase C γ was later shown to be selectively involved in the incorporation of such EtOH-up-regulated α4-$GABA_A$Rs into the plasma membrane [121]. Analogous decreases in α1- and increases in α4-containing $GABA_A$Rs have been observed in DGCs in a rat model of temporal lobe epilepsy [122, 123], later shown to involve inducible early growth response factor 3 (Egr3) up-regulation of the Gabra4 minimal promoter [124]. Consistent with the similarity of the $GABA_A$R alterations, the heightened anxiety, depression, and spatial learning deficits in epilepsy models [125–128] are also observed in rodent FAE models [60, 64, 80, 129, 130]. Collectively, these studies provide a strong link between altered $GABA_A$R function and the cognitive and behavioral abnormalities associated with FAE.

As with neurobehavioral deficits, the FAE-induced changes in $GABA_A$R responsiveness to zolpidem and THIP were also prevented by DHM co-administration with EtOH in pregnant rats. Importantly, DHM administration alone in pregnant rats had no adverse effect on litter size, progeny weight, performance in the EPM, EtOH-induced LORR, PTZ-induced seizure threshold, or DGC $GABA_A$R function. This is consistent with the previous observations that on its own, DHM is not anxiolytic, or sedative, or pro-convulsant at the dose (1 mg/kg) that blocks EtOH effects on $GABA_A$Rs in adult male rats [26]. This contrasts DHM with Ro15-4513, which was shown to block EtOH's enhancement of $GABA_A$Rs in vitro and in vivo [131, 132] and shown to also possess both pro-convulsant and anxiogenic effects due to its ability to block most $GABA_A$R subtypes [133]. DHM was also shown to greatly reduce EtOH consumption when high voluntary EtOH consumption is already established in adult male rats [26], suggesting that similar reductions in voluntary EtOH consumption could be achieved in females. The absence of adverse side effects and the ability of DHM to completely prevent FAE consequences suggest that DHM is an attractive candidate for development as a treatment for prevention of FASD.

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