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STRAIN-SPECIFIC PROGRAMMING OF PRENATAL ETHANOL EXPOSURE ACROSS GENERATIONS

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Abstract

Behavioral consequences of prenatal alcohol exposure (PAE) can be transmitted from *in utero*-exposed F1 generation to their F2 offspring. This type of transmission is modulated by genetic and epigenetic mechanism. This study investigated the intergenerational consequences of prenatal exposure to low ethanol dose (1g/kg) during gestational days 17–20, on ethanol-induced hypnosis in adolescent male F1 and F2 generations, in two strains of rats. Adolescent Long Evans and Sprague Dawley male rats were tested for sensitivity to ethanol-induced hypnosis at 3.5g/kg or 4.5g/kg ethanol dose using the loss of righting reflex (LORR) paradigm. We hypothesized that PAE would attenuate sensitivity to ethanol-induced hypnosis in the ethanol-exposed animals in these two strains and in both generations. Interestingly, we only found this effect in Sprague Dawley rats. Lastly, we investigated PAE related changes in expression of $GABA_A$ receptor α1, α4, and δ subunits in the cerebral cortex of the PAE sensitive Sprague Dawley strain. We hypothesized a reduction in the cerebral cortex $GABA_A$ receptor subunits' expression in the F1 and F2 PAE groups compared to control animals. GABAA receptor α1, α4, and δ subunits protein expressions were quantified in the cerebral cortex of F1 and F2 male adolescents by western blotting. PAE didn't alter cerebral cortical $GABA_A$ receptor subunit expressions in the F1 generation, but it decreased $GABA_A$ receptor α4 and δ subunits' expressions in the F2 generation, and had a tendency to decrease α1 subunit expression. We also found correlations between some of the subunits in both generations. These strain-dependent vulnerabilities to ethanol sensitivity, and intergenerational PAE-mediated changes in sensitivity to alcohol indicate that genetic and epigenetic factors interact to determine the outcomes of PAE animals and their offspring.

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Introduction

It is known that genetic and environmental factors are involved in the development of alcohol use disorders. Twin, linkage, candidate gene, and genome-wide association studies have all contributed to identifying genetic susceptibility factors for these disorders (see Tawa et al., 2016 for review). Environmental factors such as stress (see Varlinskaya et al., 2016 for review) and social and cultural context (Sudhinaraset et al., 2016) have also been implicated in alcohol use disorders. Furthermore, there is increasing evidence that epigenetic mechanisms modulate gene-environment interactions.

Growing evidence suggest that epigenetic modifications (histone acetylation and DNA methylation) may influence behavior. Acute alcohol exposure increases histone acetylation in rats, and anxiety-like behavior during withdrawal in chronically alcohol exposed rats. The latter effect is associated with decreased histone acetylation ({Pandey, 2008 #14}). This set of experiments also found that in mice, offspring of alcohol-treated fathers were more immobile in a force swim test then control animals. Rats sired by alcohol-treated males also showed learning deficits and impaired spatial memory (Wozniak et al. 1991). Other studies demonstrated that prenatal alcohol exposure (PAE) alters pro-opiomelanocortin gene expression and hypothalamic-pituitary-adrenal axis function via changes in DNA methylation (Gangisetty et al., 2014, Garro et al., 1991).

PAE is a leading cause of preventable birth-related defects and neurodevelopmental anomalies in the United States, with a prevalence rate of 1–5% among live births (Riley et al., 2011). PAE can induce various psychological outcomes, including an increased risk for problematic alcohol use. Interestingly, certain behavioral consequences of PAE can be transmitted from *in utero*-exposed F1 generation to their F2 and F3 offspring, as our laboratory recently demonstrated (Nizhnikov et al., 2016). Indeed, following prenatal exposure in F1, we found an increase in ethanol intake in F1 through F3 generation infant Sprague Dawley rats, and attenuated sensitivity to ethanol-induced hypnosis in the F1 and F2 generation adolescent male offspring. A possible explanation for this transgenerational transmission is through PAE-induced epigenetic modifications in the directly exposed F1 generation's epigenome, as previously demonstrated (Garro et al., 1991, Gangisetty et al., 2014). In the absence of direct exposure, transgenerational epigenetic inheritance of a phenotypic variation involves the germline transmission of altered epigenetic information (Anway et al., 2005, Skinner et al., 2010). Epigenetic reprogramming of germ lines occurs during the developmental fetal period. Primordial germ cells undergo erasure of DNA methylation as they colonize the fetus' gonads, which is followed by a re-methylation during the fetal sex determination period (Hemberger et al., 2009, Hajkova et al., 2002). Thus, exposure during the third trimester of pregnancy in rats (F0 generation) may promote epigenetic transgenerational inheritance of a specific phenotype of the prenatally exposed F1 animal, to the next generations (Skinner et al., 2010, Anway et al., 2005). Susceptibility to PAE effects and the magnitude of the changes are also largely dependent on genetic factors (Ramsay, 2010). By testing different strains of experimental animals with the same prenatal exposure conditions, we can elucidate the impact of genetics on ethanol's teratogenic properties (Chen et al., 2011, Downing et al., 2009, Sluyter et al., 2005). Chen and colleagues (2011) compared the teratogenic consequences of a single six-hour ethanol

exposure on embryonic Day 8 between three mice strains. They observed no neurodevelopmental deficits in 129S6/SvEvTac strain, while exposed DBA/2 and C57BL6/6N strains showed developmental and physiological effects. Downing and colleagues (Downing et al., 2009) also reported similar genetic susceptibility to the effect of ethanol in different mice strains. Existing knowledge concerning the influence of strain in rats is somewhat controversial (Khanna et al., 1990, Sellin and Laakso, 1987). Therefore, more research is needed to better understand strain-differences in rats, as several strains are employed in prenatal alcohol research.

PAE modulates sensitivity to ethanol-induced hypnosis. The cerebral cortex is of major importance to this behavior, as it plays a critical role in motor coordination (Donchin et al., 1998, Carlson et al., 2013, Gigante et al., 2014, Carter et al., 2016). The prenatal developmental process of the cerebral cortex is susceptible to PAE-induced disruptions that can last long into adulthood, especially when exposure occurs during late gestation (Miller, 1996, Skorput and Yeh, 2016, Miller, 1986). Therefore, assessing PAE-induced changes in the cerebral cortex may provide valuable insight into changes in motor coordination under the influence of ethanol, such as those previously reported in Sprague Dawley rats (Nizhnikov et al., 2016).

Ethanol exposure during the crucial periods of brain development disrupts γ-aminobutyric acid (GABA) transmission in the cerebral cortex and other brain regions (Barbaccia et al., 2007, Blaine et al., 1999, Allan et al., 1997, Maier et al., 1996, Hsiao et al., 1998). The GABA system, which is primarily responsible for fast neuronal inhibition within the central nervous system, plays a principal role in mediating ethanol-induced hypnosis (Kumar et al., 2009). Likewise, the developing fetal GABAergic system is a vital target for alcohol's teratogenic effects (Toso et al., 2006). A fully functional $GABA_A$ receptor complex is pentameric, consisting of a selection of five subunits from about 19 known; α (1–6), β (1–3), γ (1–3), δ, ε, ρ and θ (1–3). An individual receptor complex's subunit composition is associated with its regional location within the brain, cellular localization (synaptic or extrasynaptic), physiological functions (phasic or tonic inhibition), and kinetic and pharmacological properties (agonist and antagonists) (see reviews Lobo and Harris, 2008, Kumar et al., 2009). $GABA_A$ α1 is the most expressed subunit in the brain, making up for ~50% of all subunits, and it is primarily located on the synaptic membrane. The $GABA_A$ receptor α1 subunit's role in ethanol-induced hypnosis was previously revealed when α1 knockout mice demonstrated attenuated sensitivity to ethanol-induced hypnosis (Blednov et al., 2003a, Blednov et al., 2003b). Most of $GABA_A$ α4 and δ subunits are located on the extrasynaptic membrane area and are also involved in modulation of sensitivity to ethanol-induced intoxication via mediation of tonic currents (Jia et al., 2007, Kumar et al., 2009, Orser, 2006). Based on the roles of these subunits, variations in their expressions may contribute to PAE-related transgenerational changes in sensitivity to ethanol-induced hypnosis (Nizhnikov et al., 2016).

This study investigated the transgenerational consequences of prenatal exposure to a relatively low ethanol dose (1g/kg) during gestational days (GD) 17–20, on ethanol-induced hypnosis in adolescent F1 generation males and their unexposed F2 male adolescent offspring. We also investigated the effect of rat strain on direct and hereditary effects of low

dose PAE by testing and comparing two rat strains; the commonly used outbred Long Evans and Sprague Dawley strains. We hypothesized that PAE would attenuate sensitivity to ethanol-induced hypnosis in both rat strains and in both generations. Interestingly, we only found this effect in Sprague Dawley rats. Lastly, we investigated PAE related changes in expression of $GABA_A$ receptor α1, α4, and δ subunits in the cerebral cortex of the low-dose PAE sensitive Sprague Dawley strain. We hypothesized a reduction in the expression of $GABA_A$ receptor subunits in the cerebral cortex of the Sprague Dawley PAE group and their male offspring compared to control animals.

Methods

Animals

Adult Sprague Dawley and Long Evans rats were obtained from Taconic Biosciences (Germantown, NY, USA) and Charles River Laboratories (Wilmington, MA, USA) respectively, and were well acclimatized to our colony before breeding. These adults represented the F0 generation and were bred within strain in standard mating cages (20” x 16” x 8”), two females with one male, to produce the first filial (F1) generation. Daily vaginal smears allowed for sperm detection on copulation day designated as GD 0, as previously described (Borrow et al., 2013). This was also when females were separated from males and pair-housed. Starting on GD 17 females were single housed until birth and received intragastric (i.g) ethanol or the vehicle (water) once daily for four days, as previously described (Nizhnikov et al., 2016, Popoola et al., 2015). Litters were weaned on postnatal day (PND) 21 and siblings were same-sex pair-housed in regular sized cages (19” x 10.5” x 8”) until testing. To produce the F2 generation, adult F1 generation males and females of the same prenatal exposure groups (ethanol male with ethanol female; or water male with water female) were mated as described above. However, this time, pregnant F1 females remained undisturbed throughout pregnancy. Only male offspring were tested in this study.

The colony room was maintained at a 10–14 light-dark cycle through mating and prior to weaning, with light on at 9am and off at 7pm. The light cycle changed after weaning to 12–12hr light-dark cycle, with the light on at 12 midnight and off at 12 noon. Colony rooms’ temperature and humidity were maintained at 22°C and 40%, respectively. Food and water were provided *ad libitum* while cage enrichment or nesting materials were not provided. No animal was used for more than one experiment, and a maximum of two subjects per litter were used in each experiment. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Binghamton University.

Prenatal Exposure

From GD 17–20, pregnant F0 generation rats received 1g/kg dose of 12.6% v/v ethanol solution in water, or the equivalent volume of water once daily via intragastric administration as previously described (Nizhnikov et al., 2016, Popoola et al., 2015). In summary, the solution was delivered with a 10mL syringe into the subject’s stomach via a polyethylene (PE 50) tube mounted on a 21-G needle. This procedure was conducted

between 10 and 11 am daily in a designated holding room, and lasted for approximately 15–20 seconds per subject.

Loss of Righting Reflex

On PND 42 and starting at approximately 7am, 159 adolescent males were tested for sensitivity to ethanol-induced hypnosis as previously described (Nizhnikov et al., 2016). In summary, subjects received a 3.5g/kg or 4.5g/kg dose of 20% v/v ethanol solution in 0.9% saline via intraperitoneal (i.p.) administration. These were selected since our previous findings (Nizhnikov et al., 2016) revealed dose-dependent effects of PAE at these two doses. Animals were then observed in their cage until they lost the capacity to return to their four paws when placed on their backs (i.e. righting reflex). Following this loss of righting reflex (LORR), subjects were maintained on their backs in a v-shaped trough until they regained their righting reflex and demonstrated it three times within 60 seconds. The latency to LORR was defined as the duration between ethanol administration and LORR, while the duration between the loss and regain of righting reflex was designated as the LORR duration.

Blood Ethanol Concentration

Immediately following regain of righting reflex, subjects were rapidly decapitated and trunk blood was collected in a vaccutainer coated with ethylenediaminetetraacetic acid (EDTA; BD, Franklin Lakes, NJ). Plasma was then extracted from the blood by centrifugation at 4°C and 1500g for 15 minutes. Blood ethanol concentration (BEC) was analyzed using an AM1 alcohol analyzer (Analox Instruments, Lunenburg, MA).

$GABA_A$ subunits expression

This experiment investigated the effect of prenatal ethanol exposure on $GABA_A$ subunits' expression in Sprague Dawley rats. Fifty-three adolescent male subjects that were postnatally experiment-naive, were rapidly decapitated on PND 42. Their brains were harvested and flash frozen in 2-methyl butane (Sigma-Aldrich, St. Louis, MO) on dry ice, and stored at -80 °C. Using L.W. Swanson's atlas (3rd Edition, Elsevier Inc., 2004) as a guide, both hemispheres of the cerebral cortex were dissected on ice in a petri dish containing phosphate buffered saline (PBS) and harvested. Tissue from each sample was homogenized with a XL-2000 series sonicator (Qsonica LLC, Newtown, CT) in buffer (10g Sodium dodecyl sulfate (SDS), 2mL 0.5M EDTA, 10mL 1M Tris and 1L dH_2O) after which, protein concentrations were quantified using a Pierce Bicinchoninic acid (BCA) protein assay kit (Thermoscientific, Rockford, IL). Subsequently, 50 ug of protein per sample was loaded into precast tris-glycine electrophoresis gels (Invitrogen, Carlsbad, CA) and run for 120–150 minutes at 125 volts and 500 mA. Proteins were transferred onto polyvinyl diflouride membrane (included in the kit) for 7 minutes, using the iBlot gel dry-transfer equipment and transfer kit (Invitrogen, Carlsbad, CA). Membranes were blocked overnight in ~10 mL bovine serum albumin (BSA) (1g BSA per 100 mL TBS-1%Tween) and probed with anti-$GABA_A$ receptor α1 and δ (Novus Biologicals, Littleton, CO.), and α4 (Millipore, Lake Temecula, CA) subunit proteins primary antibodies (α1 - 1:1000; α4 - 1:500 and δ - 1:500). Membranes were subsequently incubated in the appropriate anti-goat (1:10000) or anti-rabbit (1:10000) secondary antibody obtained from Thermoscientific (Waltham, MA).

Protein bands were detected with enhanced chemiluminescence (GE Healthcare, Piscataway, NJ), and visualized by exposure to photographic film (Bioexpress, Kaysville, UT). Beta-actin (Millipore, Lake Temecula, CA) was used to control for equivalent protein quantity loading across gel wells and samples. Antibodies were diluted in blocking buffer.

Final optical density (OD) was computed per sample as a ratio of target protein OD to β-actin OD (designated as normalized OD) and expressed as a percentage of the average normalized OD from water-control loaded rats ((Protein OD/ Protein β-Actin) / (Average (Control OD/ Control β-Actin))*100).

Statistical Analysis

Latency to LORR, LORR duration, and BEC were analyzed by dose and within filial generations by a 2-way ANOVA (prenatal exposure x strain). As the main goal of this experiment was to investigate the effect within strain, when the 2-Way ANOVA revealed a trend or a significant interaction between prenatal exposure and strain at an ethanol dose, a 1- way ANOVA was further conducted within strains. $GABA_A$ receptor subunits protein expression from western blot was not normally distributed. Therefore, we log-transformed data, then analyzed it using 1-way ANOVAs. Pearson Correlations were used to investigate the relationships between $GABA_A$ receptor subunits protein expressions. A p-value less than 0.05 was considered significant, while p-values greater than 0.05 but less than 0.1 were reported as a trend towards significance. All analyses were conducted with SPSS v20 analysis software (IBM, Armonk, NY). All data are presented as mean ± S.E.M.

RESULTS

Adolescent Sensitivity to Ethanol's Hypnotic Properties

The LORR test was used to investigate the transgenerational effect of prenatal ethanol exposure on sensitivity to ethanol-induced hypnosis in adolescent (PND 42) Sprague Dawley and Long Evans male rats. Two doses of ethanol were tested (3.5 and 4.5 g/kg) in animals exposed to ethanol or water *in utero* (F1) and their offspring (F2).

F1 Generation

Latency to Loss of Righting Reflex—At the 3.5g/kg ethanol dose, a 2-way ANOVA revealed a significant main effect of strain ($F_{1, 49} = 7.64$; $p<0.01$), while there was no effect of prenatal exposure or interaction between prenatal exposure and strain. Long Evans rats had longer latency to LORR compared to their Sprague Dawley counterparts (See Figure 1: Left).

At the 4.5g/kg ethanol dose, a 2-way ANOVA also revealed only a trend for an effect of strain ($F_{1, 35} = 3.62$; $p=0.07$), but no effect of prenatal exposure or significant interaction between prenatal exposure and strain was found. Like the lower dose, the 4.5g/kg ethanol dose also produced a trend for a longer latency to LORR in Long Evans compared to Sprague Dawley adolescent male rats (See Figure 1: Right).

Loss of Righting Reflex Duration—At the 3.5g/kg ethanol dose, a 2-way ANOVA revealed significant main effects of prenatal exposure ($F_{1, 49} = 8.54$; $p<0.01$), strain ($F_{1, 49} = 59.95$; $p<0.001$), and a significant interaction between prenatal exposure and strain ($F_{1, 49} = 14.98$; $p<0.001$). LORR duration was longer in Sprague Dawley males compared to their Long Evans counterparts (Figure 2: Left). In addition, ANOVAs within strain revealed that prenatal exposure had a significant effect on LORR duration in Sprague Dawley animals ($F_{1, 33} = 26.55$; $p<0.001$), but not in Long Evans. In the Sprague Dawley strain, LORR duration was shorter in prenatally ethanol-exposed males compared to the water-exposed animals (Figure 2: Left).

At the 4.5g/kg ethanol dose, a 2-way ANOVA revealed a significant main effect of strain ($F_{1, 35} = 20.17$; $p<0.001$), but no main effect of prenatal exposure or interaction between prenatal exposure and strain. Again, LORR duration was longer in Sprague Dawley compared to Long Evans rats (Figure 2: Right).

Blood Ethanol Concentration—At the 3.5g/kg ethanol dose, a 2-way ANOVA analysis of the BEC at awakening revealed a significant main effect of strain ($F_{1, 49} = 4.96$; $p<0.05$), but no effect of prenatal exposure. As shown in Figure 3 (Left), Long Evans rats had higher BEC at awakening compared to Sprague Dawley rats. Additionally, the 2-way ANOVA revealed a trend toward a significant interaction between prenatal exposure and strain ($F_{1, 49} = 3.85$; $p = 0.056$). One-way ANOVAs performed within strain revealed that the interaction was driven by a significant main effect of prenatal exposure in the Sprague Dawley strain ($F_{1, 33} = 11.63$; $p < 0.005$), while the Long Evans strain showed no prenatal exposure effects (Figure 3; Left).

At the 4.5g/kg ethanol dose, a 2-way ANOVA revealed a main effect of strain ($F_{1, 35} = 18.40$; $p < 0.001$), but no main effect of prenatal exposure. As shown in Figure 3 (Right), Long Evans rats regained their righting reflex at higher BECs compared to Sprague Dawley animals. There was also a significant interaction between prenatal exposure and strain ($F_{1, 35} = 4.34$; $p < 0.05$). A one-way ANOVA further revealed a main effect of prenatal exposure on BEC in Sprague Dawley adolescent male offspring ($F_{1, 23} = 5.106$; $p < 0.05$), but not in Long Evans rats (Figure 3; Right).

F2 Generation

Latency to Loss of Righting Reflex—At the 3.5g/kg ethanol dose, a 2-way ANOVA revealed no significant effect of strain, exposure or interaction between exposure and strain in adolescent male offspring of prenatally exposed Long Evans and Sprague Dawley rats (Figure 4: Left).

Similarly, at the 4.5g/kg ethanol dose, a 2-way ANOVA revealed no significant effect of strain or exposure group, and no significant interaction between exposure and strain (Figure 4: Right).

Loss of Righting Reflex Duration—At the 3.5g/kg ethanol dose, a 2-way ANOVA revealed significant main effects of exposure group ($F_{1, 39} = 4.28$; $p < 0.05$), strain ($F_{1, 39} = 17.21$; $p < 0.001$), and a trend toward significant interaction between exposure group and

strain ($F_{1, 39}$ = 2.92; p = 0.09). Sprague Dawley rats demonstrated longer LORR duration compared to their Long Evans counterparts (Figure 5: Left). ANOVAs within strains revealed that exposure had a significant main effect on LORR duration in Sprague Dawley rats ($F_{1, 25}$ = 8.13; p < 0.01), but not in Long Evans animals. Indeed, in the Sprague Dawley offspring of prenatally ethanol-exposed animals, a shorter LORR duration was observed compared to the water group (Figure 5: Left).

At the 4.5g/kg ethanol dose, a 2-way ANOVA revealed a significant effect of strain ($F_{1, 32}$ = 7.78; p < 0.01), as Long Evans rats had a shorter LORR duration than Sprague Dawley rats. Neither an effect of exposure group nor a significant interaction between exposure and strain was found (Figure 5: Right).

Blood Ethanol Concentration—At the 3.5g/kg ethanol dose, a 2-way ANOVA of the BEC at awakening revealed a significant effect of strain ($F_{1, 39}$ = 23.04; p < 0.001), a trend toward a significant effect of exposure group ($F_{1, 39}$ = 3.17; p = 0.08), and no significant interaction between exposure and strain. Long Evans rats had higher BEC at awakening compared to Sprague Dawley rats. ANOVAs within strain further revealed an effect of exposure group only in Sprague Dawley offspring ($F_{1, 25}$ = 5.01; p < 0.05), as ethanol-exposed (F2) Sprague Dawley awaken with lower BEC (Figure 6: Left).

At the 4.5g/kg ethanol dose, a 2-way ANOVA revealed no significant effect of strain or exposure group, and no significant interaction between exposure and strain (Figure 6: Right).

$GABA_A$ Receptor Subunits Protein Expression

We investigated the intergenerational effect of prenatal ethanol on the $GABA_A$ subunits' expression in the cerebral cortex of Sprague Dawley adolescent (PND 42) rats exposed to ethanol or water *in utero* (F1) and their experimentally-naive offspring (F2). This was to elucidate the role of GABAergic transmission in the observed alteration in sensitivity to ethanol-induced hypnosis in the Sprague Dawley strain.

F1 Generation—An ANOVA revealed no main effect of prenatal exposure on $GABA_A$ α1, α4 and δ subunits (Fig. 7). Interestingly, we found a correlation between α1, and δ subunits protein expression (r=0.74, n=13, p< .005).

F2 Generation—In the F2 generation, there was significant main effects of treatment on $GABA_A$ α4 subunit ($F_{1, 17}$ = 6.34; p < 0.05) and δ ($F_{1, 18}$ = 4.35; p < 0.05), while the difference in α1 ($F_{1, 17}$ = 4.21; p = 0.06) subunits only approached significance (Fig. 7). We also found a correlation between α1, and δ subunit protein expression (r=0.77, n=18, p< .001), in this generation.

Discussion

The results of the present study demonstrate that the programming of decreased sensitivity to ethanol-induced hypnosis by prenatal ethanol exposure is strain specific. Therefore, gene-environment interactions are necessary for the establishment of attenuated sensitivity to

ethanol-induced hypnosis and its maintenance into the next generation of male offspring. In the present study, we replicated our previous findings that prenatal ethanol exposure programs sensitivity to acute 3.5g/kg ethanol-induced hypnosis in F1 Sprague Dawley rats and their unexposed F2 generation (Nizhnikov et al., 2016), but the effect was not seen in Long Evans rats. Furthermore, ethanol treatment during gestation modulated $GABA_A$ receptor subunit expression, particularly the α4 and δ subunits, in the Sprague Dawley F2 generation.

In the present study, low-dose ethanol exposure between GD 17–20 decreases the duration of acute 3.5 g/kg ethanol dose-induced hypnosis in adolescent male Sprague Dawley rats, when compared to their prenatal water-treated counterparts. This decrease was associated with a higher BEC at awakening compared to control, indicating that the difference between the treatment groups may be attributed to variations in sensitivity to the ethanol's hypnotic effect and not its pharmacokinetics. Although the duration of hypnosis at 4.5 g/kg dose was not significantly different between the two treatment groups, the Sprague Dawley PAE animals still had higher BEC at awakening, suggesting their lower sensitivity to ethanol at this dose as well. Sensitivity to ethanol-induced hypnosis is strongly associated with alcohol-consumption patterns. Various studies have demonstrated that increased alcohol consumption is associated with attenuated sensitivity to alcohol-induced hypnosis (Naassila et al., 2002, Thiele et al., 2000). Therefore, attenuated sensitivity following PAE, as we observed, may be relevant to the increased risk for problematic alcohol- and psychoactive drugs-use as reported in clinical PAE cases (Baer et al., 2003, Pfinder et al., 2014, Baer et al., 1998). Indeed, we previously found that Sprague Dawley PAE infants consume more ethanol when compared to water-treated and non-manipulated groups (Nizhnikov et al., 2016).

Prenatally water- and ethanol-exposed animals were mated and their male offspring were also tested for sensitivity to ethanol-induced hypnosis. In this second (F2) generation, results were very similar to what we found in the prenatally exposed F1 generation. Indeed, ethanol exposure programmed sensitivity to the 3.5 g/kg ethanol dose only in the F2 generation of Sprague Dawley rats, and not in Long Evans animals. This effect in the Sprague Dawley strain was also associated with higher BEC at awakening in the ethanol-treated F2 generation. This finding also replicates our previous report that low-dose PAE-induced attenuation in sensitivity to ethanol-induced hypnosis can be transmitted to the F2 generation male offspring (Nizhnikov et al., 2016).

Contrary to our hypothesis, Long Evans rats were not vulnerable to the programming effect of a low dose PAE (F1 generation). Furthermore, the prenatal treatment did not affect the F2 generation of male offspring. We found no significant effect of PAE on sensitivity to ethanol-induced hypnosis in terms of latency, LORR duration or BEC at awakening in this strain. This resilience in Long Evans rats was unexpected as other studies have reported both ethanol use-related and–unrelated behavioral effects of PAE in Long Evans rats (Barbier et al., 2009, Hamilton et al., 2014). Barbier and colleagues (2009) reported attenuated LORR duration by PAE in this strain, although their dams consumed 10% ethanol as the sole drinking fluid throughout gestation and lactation. Such higher-dose and longer exposure may distinguish their results from ours, as we employed 1g/kg daily dose for only four days. This

suggests that a larger ethanol dose given to dams (F0) may have been needed to induce the epigenetic effect associated with intergenerational transmission of attenuated sensitivity to ethanol-induced hypnosis in the Long Evans strain.

Interestingly, our results also showed that Long Evans rats are less sensitive to the hypnotic effect of ethanol than Sprague Dawley rats. Indeed, both PAE and water-exposed Long Evans rats (F1) showed a longer latency to LORR at 3.5 and 4.5 g/kg ethanol doses, compared to Sprague Dawley rats. This effect was not found in the F2 generation. The reason for a lack of a similar effect of treatment in the F2 is unclear. Latency to LORR is a measure that is often described as markers of sensitivity, although less often than duration of LORR. Variation in latency to LORR has been suggested to be modulated by brain catalase activity (Swartzwelder, 1984; Correa, M et al. 2001). Many studies report a lack of similarity between the effect of alcohol on latency and duration of LORR (Ozburn et al. 2013, Correa, M et al. 2001, Walls et al. 2012). In the present study, LORR duration revealed that Long Evans F1 and F2 generations showed a lower sensitivity to ethanol at both 3.5 and 4.5 g/kg ethanol doses. In this strain, the F1 generation's BEC at awakening was significantly greater than the BECs of Sprague Dawley animals at both 3.5 and 4.5 g/kg ethanol doses, but only at the 3.5 g/kg dose in the F2 generation. The lack of effect of prenatal exposure may be linked again to a lower sensitivity to ethanol.

Strain-dependent differences in both, sensitivity to alcohol and teratogenic effects of PAE, are important phenomena that several studies have investigated (Cailhol and Mormede, 2001, Blednov et al., 2005, Kempf et al., 1985, Moore et al., 2010). Our finding corroborates existing reports of strain-dependent differences in vulnerability to ethanol's behavioral and teratogenic effects in rodents (Chen et al., 2011, Downing et al., 2009, Sluyter et al., 2005). For instance, 129S6/SvEvTac mouse strain is less vulnerable to PAE compared to C57BL/6N and DBA/2 strains. Vulnerability to the effects of a large dose of ethanol during pregnancy was observed as PAE Sprague Dawley male offspring displayed more hippocampus-dependent behavioral deficits compared to Brown Norway rats (Sittig et al., 2011). Interestingly, several studies have found discrepancies in their results when investigating rat strain differences in sensitivity to ethanol. For example, using ethanol-induced hypothermia and motor impairment with the tilting plane test, Khanna et al (1990) found no difference in acute ethanol sensitivity between several strains of rats including Wistar, Sprague-Dawley and Long Evans. Using this same test, others (Sellin and Laakso, 1987) found that ethanol was more effective in inducing motor impairment in the Long Evans strain compared to Wistar rats (they did not test Sprague Dawley rats). Also, (Cailhol and Mormede, 2001) found a strain difference in ethanol self-administration. Our results, however, are in line with existing evidence (Horowitz et al., 1999), indicating that Long Evans rats consume more ethanol than Sprague Dawley rats, and are less behaviorally sensitive to ethanol and cocaine induced behavioral challenges when co-ingested (Whishaw et al., 2003). Together, our strain-dependent effect in the present study adds to the previous literature, suggesting strain-specific vulnerability to alcohol-induced behavior.

Although we report that Long Evans rats are less sensitive to ethanol-induced hypnosis, and they consume more ethanol than Sprague Dawley as previously described (Horowitz et al., 1999), it remains questionable whether either of these two strains is more vulnerable to

alcohol use disorder (AUD) than the other. Further assessments of strain-differences in other characteristics of AUD such as ethanol's anxiolytic and rewarding effects are still necessary to fully distinguish strain-dependent vulnerability to AUD between both strains.

Epigenetic mechanisms have been shown to be involved in PAE effects in the developing offspring (see Ungerer et al., 2013 for review). We hypothesize that developmental exposure to ethanol may trigger epigenetic modifications in the Sprague Dawley F1 generation, which then mediates attenuated sensitivity. Subsequently, such epigenetic modifications may be transmitted from the F1 to F2 generation as previously described (Anway et al., 2005, Pfinder et al., 2014). Sensitivity to alcohol is a quantitative and qualitative trait that is determined by a complex integration of several genes (genetics) and their interaction with the environment (epigenetics) (Morozova et al., 2014). Approximately 30% to 70% of vulnerability to drug abuse is genetically heritable (Goldman et al., 2005), while environmental factors account for the rest (Renthal and Nestler, 2008), supposedly through epigenetic mechanisms., Therefore, both genetic and epigenetic factors may contribute to the strain-differences in vulnerability to PAE and ethanol-induced hypnosis between Long Evans and Sprague Dawley strains. Clearly, Sprague Dawley and Long Evans strains are genetically different. In the present study, PAE constitutes an environmental factor that interacts with the rat strain, a genetic factor, to program sensitivity to acute ethanol-induced hypnosis. Therefore, genetic differences between strains may underpin the differences in vulnerability to PAE-induced sensitivity to ethanol-induced hypnosis.

Maternal care may constitute an additional environmental factor contributing to these strain differences. Variations in maternal care demonstrated by these two strains were previously characterized (Popoola et al., 2015). These variations may contribute an additional epigenetic component to strain-difference in sensitivity to ethanol-induced hypnosis. Quantity and quality of early-life maternal care is known to program long-term neurophysiology and behavior via epigenetic mechanisms such as DNA methylation and histone acetylation (Bagot et al., 2012, Champagne, 2008, Cameron et al., 2005, Cameron, 2011). One possible epigenetic target may be the GABAergic system. In particular, the $GABA_A$ receptor is well known for its role in modulation of the effect of ethanol (Blednov et al., 2003a). Furthermore, natural variations in maternal care can program $GABA_A$ receptor expression (Caldji et al., 2003, Caldji et al., 2004). Therefore, strain-differences in maternal care could contribute significantly to the differences in sensitivity to ethanol-induced hypnosis between the strains.

We investigated the programming effects of PAE on the $GABA_A$ receptor subunits protein expressions in the Sprague Dawley strain that demonstrated PAE-induced behavioral effects in the present study. We quantified α1, α4, and δ subunits in the cerebral cortex, the motor coordinating area of the brain (VandenBerg et al., 2002) and a critical target for PAEinduced teratogenesis (Skorput and Yeh, 2016). We predicted a reduction in $GABA_A$ α1, α4 and δ subunits' expression in PAE animals and their F2 generation offspring. We found that offspring of prenatally ethanol-exposed animals (F2 generation) had a significant decrease in $GABA_A$ α4 and δ subunits protein expression, and a trend for less α1 subunit protein expression, compared to offspring prenatally exposed to water. However, no change in the F1 generation was observed. This result suggests that changes in these receptors, at least in

the F1 generation, are not needed for a reduction in sensitivity to the hypnotic effects of ethanol. However, one possible, explanation for these results could be that there was an interaction between prenatal ethanol exposure and the intragastric intubation handling process, that was performed in both the control and ethanol-treated groups in the F1 generation. This could have increased the variability in this generation, and masked the effect of ethanol-treatment, particularly in the δ subunit expression. This variability in receptor subunits' expression in the F1 generation, decreases the probability to identify permanent changes in protein expression that can be transmitted to the next generation. As the second generation did not get direct ethanol or water exposure through gavage, the F2 generation showed less variability in protein expression compared to the F1 generation as can be seen in the differences in Standard Deviation (S.D.: α1, F1: 50.21; F2: 31.83; α4, F1: 75.88; F2: 36.22; δ, F1: 119.03; F2: 39.44). Although this is highly speculative, we suggest that changes in protein expressions necessary for the inheritance of the attenuated sensitivity to ethanol-induced hypnosis are revealed in the F2 generation due to a decrease in variability. Experiments using multiple exposure techniques (such as intraperitoneal, vapor, intragastric) should be used to investigate their effects on GABAA protein expressions in the brain and in subsequent generations.

Many $GABA_A$ subunits have been implicated in the behavioral response to acute ethanol exposure, suggesting critical involvement of $GABA_A$ receptors in the expression of ethanol-induced behavior. An acute alcohol exposure is sufficient to alter $GABA_A$ subunit composition, particularly α1 and α4 subunit expressions (Liang et al. 2007). The α1 subunit has been shown to be involved in ethanol-induce sedation (Lobo, 2008). The role of α4, particularly in the cerebral cortex in ethanol-induced behavior, is not well understood, and its limited expression within the cortex also limits the understanding of our results (Wisden et al., 1991). An important fraction of α4 subunits normally co-assembles with δ subunits to form extra-synaptic receptors (Sun et al. 2004). Extra-synaptic $GABA_A$ receptors containing the δ subunit are enhanced by low concentration of ethanol (Wallner et al. 2003). However, Borghese et al. (2006) found no effects of low ethanol concentration on tonic dentate gyrus currents mediated by α4 and δ subunits. Therefore, there is still some controversy about the effect of ethanol on $GABA_A$ subunit activity. Nevertheless, we are the first to report changes in $GABA_A$ receptor subunits expression in offspring of PAE animals.

The lower expressions of α4 and δ subunits in the F2 ethanol-exposed group compared to the control group, suggest a decrease in extra-synaptic GABAA receptors which may contribute to the shorter LORR duration in this group (Blednov et al., 2003a). On the other hand, the absence of significant changes in $GABA_A$ receptor subunit protein expressions in our F1 generation, despite the behavioral effect, also suggest that these subunits are not vital to PAE-related differences in sensitivity to ethanol-induced hypnosis.

Our results revealed a strong correlation between $GABA_A$ α1 and δ subunits' expressions within the cerebral cortex in both F1 and F2 generations of male rats. This suggest that the two subunits are co-localized in the cortex. It has been suggested that these two subunits differ in their location (Araujo et al. 1998; Quirk et al. 1995). Interestingly, research from Glykys et al. (2007) demonstrated that α1δ assembly exist in mice hippocampal interneurons. These $GABA_A$ α1δ assemblies reportedly mediate tonic inhibitory current,

which is an intrinsic property of delta-containing $GABA_A$ receptors, and ethanol significantly potentiates such α1δ-mediated current (Glykys et al. 2007). These evidence suggest that α1 and δ subunits may synergistically modulate ethanol-induced potentiation of inhibitory current, and the resultant behavior such as hypnosis. We hypothesize that this is the case in our F2 generation, where correlation between α1 and δ expressions in the PAE group may complement significantly reduced α4 and δ xpression to mediate attenuated sensitivity to ethanol-induced hypnosis. The presence of the same strong correlation between α1 and δ expression in our F1 generation, as in F2, may also contribute to the attenuated sensitivity to ethanol-induced hypnosis in the F1 PAE group.

We only investigated changes in subunits expression in the cerebral cortex, while other brain areas such as the cerebellum and striatum may also play important roles and will need to be investigated. Further studies should also include an un-manipulated control group to verify the effect of the gavage procedure on $GABA_A$ receptor protein expressions. Other potential candidate mechanisms and bio-molecular markers should also be investigated to better understand the mechanisms that mediate PAE-related differences in sensitivity to ethanol-induced hypnosis.

One limitation of the study is that the two strains (F0 generation) were purchased from different vendors. This difference in the F0 generation could have contributed to some of the differences in behavior that we have observed. However, maternal care is very sensitive to environmental stressors (Cameron et al.,2008) and as we have found no significant difference between the F0 and F1 un-manipulated dams in both strains (Popoola et al. 2015), this suggest that the origin of the F0 animals does not contribute significantly to the present experimental effects. Furthermore, as previous studies (Chen et al., 2011, Downing et al., 2009) have observed strain differences between strains whose progenitors were reared under the same facility conditions, we believe that our findings are not due to different originating colony conditions of our progenitor F0 generation adults.

It is also important to note that the present experiments were only performed in males. This was mainly due to our need for more females than males for breeding and the production of the F2 generation. Future studies should investigate the intergenerational effects of PAE in females, as maternal inheritance may contribute into developmental consequences on males and females F2 offspring.

Conclusion

This study investigated the intergenerational consequences of prenatal exposure to low ethanol dose during late gestation on ethanol-induced hypnosis. Our results demonstrate that PAE strain-dependently attenuates sensitivity to ethanol-induced hypnosis during adolescence. Furthermore, we validate strain-differences in sensitivity to ethanol-induced hypnosis between Long Evans and Sprague Dawley rat strains. We also provide evidence that certain $GABA_A$ receptor subunits may, at least in part, be responsible for the PAE-related attenuation of sensitivity to ethanol-induced hypnosis. However, this does not eliminate the possible roles of other subunits in other brain areas not tested in this study. Most importantly, these findings suggest that initial susceptibility to ethanol's teratogenic effects and the eventual outcomes depend on an interaction between genetic background and

environmental factors. Such environmental factors may include the intensity of the prenatal alcohol insult, maternal care and stress. Increasing knowledge of the important impact of environmental factors such as maternal care and environmental enrichment continue to provide valuable insight into improving diagnosis and treatment of fetal alcohol spectrum disorders (Hannigan and Berman, 2000, Peadon et al., 2009, Kalberg and Buckley, 2007, Streissguth et al., 2004). Lastly, the strain differences that we report in this study emphasize the need to carefully select animal models in PAE research, based on the exposure paradigm of choice, and PAE-related outcomes of interest. Based on our findings and existing literature, some strains may be more responsive to mild ethanol-exposure paradigms, while others require greater exposure.

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Highlights

- Gestational alcohol exposure strain-specifically affects alcohol sensitivity in two generations.
- Gestational alcohol exposure modulates GABAA receptor subunits expression in grant-offspring.
- The effects of gestational alcohol exposure are gene x environment interaction dependent.

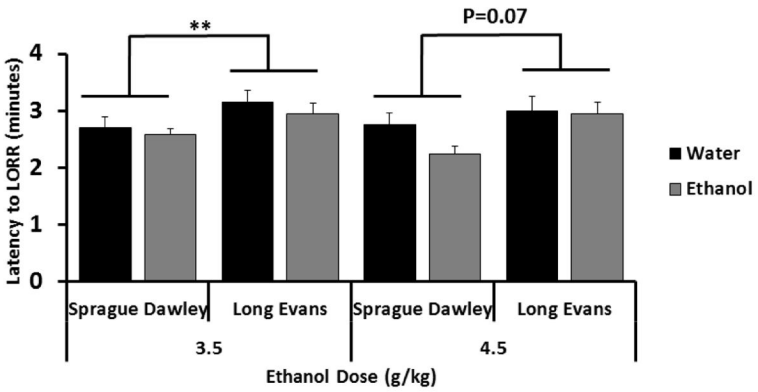

Figure 1.

F1 Generation latency to lose righting reflex (LORR). No effect of exposure was found. Long Evans (3.5g/kg, water: n=7; ethanol: n=9; 4.5g/kg, water: n=5; ethanol: n=7) demonstrate a longer latency compared to Sprague Dawley at 3.5g/kg dose (water: n=10; ethanol: n=24) and a trend at the 4.5g/kg dose (water: n=8; ethanol: n=16). Data presented as Mean + S.E.M (**$p < 0.01$).

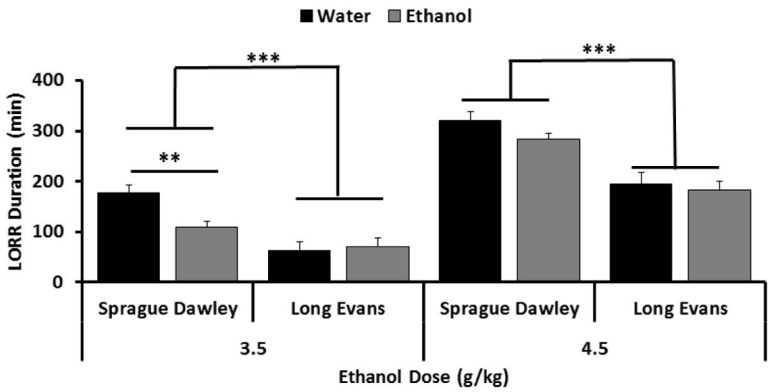

Figure 2.

F1 Generation loss of righting reflex (LORR) duration. In the Sprague Dawley rats, Ethanol group demonstrate shorter LORR duration compared to the Water group at 3.5g/kg (water: n=10; ethanol: n=24) but not 4.5g.kg dose (water: n=8; ethanol: n=16). No effect of exposure was found in Long Evans rats (3.5g/kg, water: n=7; ethanol: n=9; 4.5g/kg, water: n=5; ethanol: n=7). At both doses, LORR duration in Long Evans was shorter compared to Sprague Dawley. Data presented as Mean + S.E.M (**p < 0.01, ***p < 0.001).

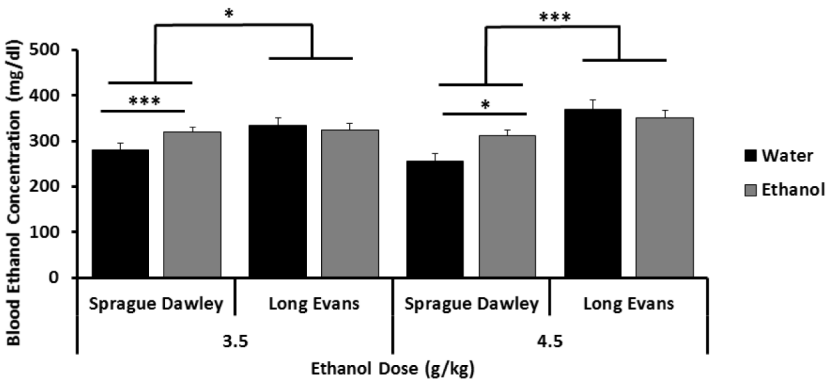

Figure 3.

F1 Generation blood ethanol concentration (BEC) at awakening. In the Sprague Dawley rats, Ethanol group regain their righting reflex at a higher BEC compared to Water group at both 3.5g/kg (water: n=10; ethanol: n=24) and 4.5g.kg doses (water: n=8; ethanol: n=16). No effect of exposure was found in Long Evans rats (3.5g/kg, water: n=7; ethanol: n=9; 4.5g/kg, water: n=5; ethanol: n=7). Long Evans rats also regain righting reflexes at higher BEC compared to Sprague Dawley counterparts at both doses. Data presented as Mean + S.E.M (*$p < 0.05$, ***$p < 0.005$)

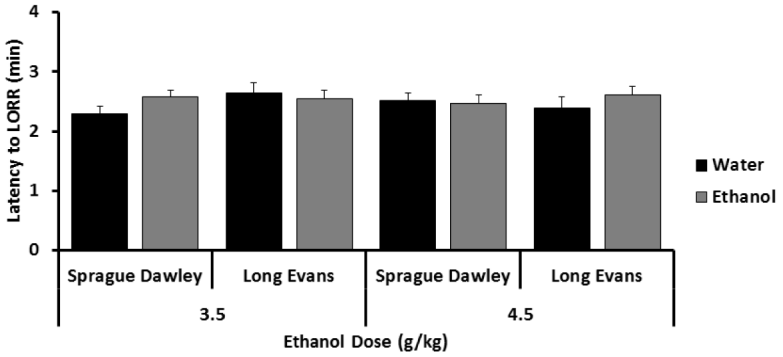

Figure 4.

F2 Generation Sprague Dawley (3.5g/kg, water: n=12; ethanol: n=14; 4.5g/kg, water: n=10; ethanol: n=9) and Long Evans (3.5g/kg, water: n=6; ethanol: n=8; 4.5g/kg, water: n=5; ethanol: n=9) rats' latency to the lose righting reflex (LORR). There were no significant differences between treatment groups or strains. Data presented as Mean + S.E.M.

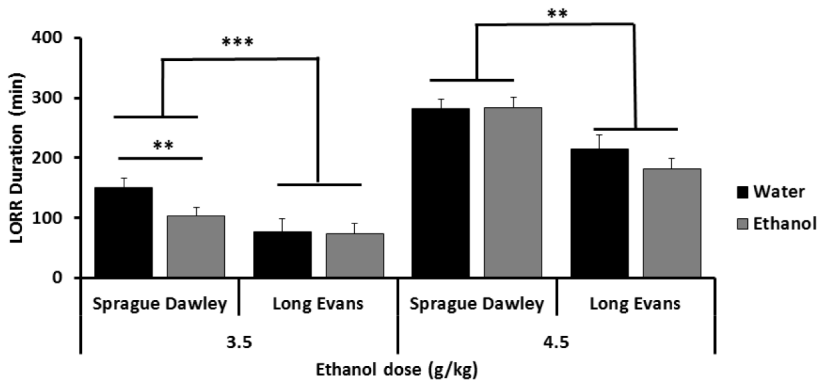

Figure 5.

F2 Generation Loss of righting reflex (LORR) duration. In the Sprague Dawley rats (3.5g/kg, water: n=12; ethanol: n=14; 4.5g/kg, water: n=10; ethanol: n=9), Ethanol group demonstrate shorter a LORR duration compared to the Water group at 3.5g/kg but not 4.5g.kg dose. No effect of exposure was found in Long Evans rats (3.5g/kg, water: n=6; ethanol: n=8; 4.5g/kg, water: n=5; ethanol: n=9). At both doses, LORR duration in Long Evans was shorter compared to Sprague Dawley. Data presented as Mean + S.E.M (**p < 0.01, ***p < 0.001).

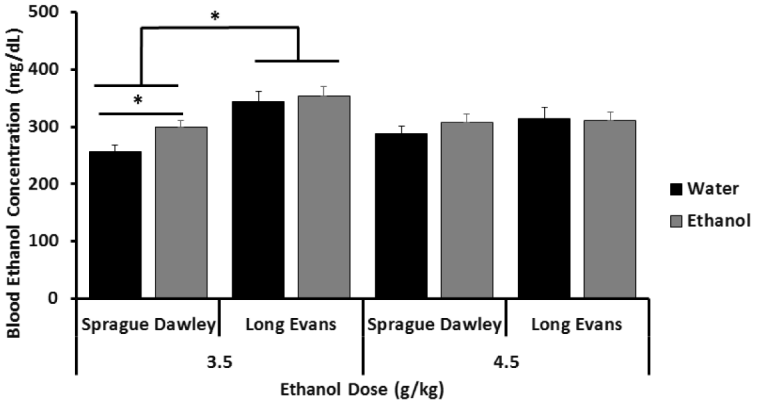

Figure 6.

F2 Generation blood ethanol concentration (BEC) at awakening. Sprague Dawley Ethanol group regained their righting reflex at a higher BEC compared to Water group at both 3.5g/kg (water: n=12; ethanol: n=14) but not 4.5g.kg dose (water: n=10; ethanol: n=9). No effect of exposure was found in Long Evans rats (3.5g/kg, water: n=6; ethanol: n=8; 4.5g/kg, water: n=5; ethanol: n=9). Long Evans rats also regain righting reflexes at higher BEC compared to Sprague Dawley counterparts at 3.5g/kg but not 4.5g/kg. Data presented as Mean + S.E.M (*$p < 0.05$).

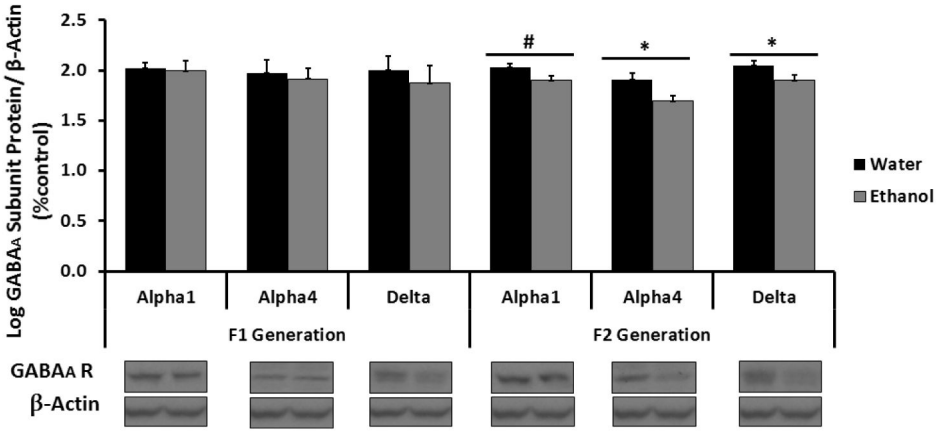

Figure 7.

GABAA α1, α4, and δ subunit expressions in the cerebral cortex of adolescent Sprague Dawley rats (F1, water: n=6; ethanol: n=7; F2, water: n=10; ethanol: n=9). No effect of treatment in the F1 generation. In F2 generation, ethanol group express less α4 and δ subunits than water and a trend towards less α1 subunit. Data presented as Mean ± S.E.M (*$p < 0.05$).