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Effects of Ethanol Exposure In Utero on Cajal–Retzius Cells in the Developing Cortex

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Abstract

Background—Prenatal exposure to ethanol exerts teratogenic effects on the developing brain. Here, we tested the hypothesis that exposure to ethanol in utero alters the disposition of Cajal–Retzius cells that play a key role in orchestrating proliferation, migration, and laminar integration of cortical neurons in the embryonic cortex.

Methods—Pregnant Ebf2-EGFP mice, harboring EGFP-fluorescent Cajal–Retzius cells, were subjected to a 2% w/w ethanol consumption regimen starting at neural tube closure and lasting throughout gestation. Genesis of Cajal–Retzius cells was assessed by means of 5-bromo-2-deoxyuridine (BrdU) immunofluorescence at embryonic day 12.5, their counts and distribution were determined between postnatal day (P)0 and P4, patch clamp electrophysiology was performed between P2 and P3 to analyze GABA-mediated synaptic activity, and open-field behavioral testing was conducted in P45–P50 adolescents.

Results—In Ebf2-EGFP embryos exposed to ethanol in utero, we found increased BrdU labeling and expanded distribution of Cajal–Retzius cells in the cortical hem, pointing to increased genesis and proliferation. Postnatally, we found an increase in Cajal–Retzius cell number in cortical layer I. In addition, they displayed altered patterning of spontaneous GABA-mediated synaptic barrages and enhanced GABA-mediated synaptic activity, suggesting enhanced GABAergic tone.

Conclusions—These findings, together, underscore that Cajal–Retzius cells contribute to the ethanol-induced aberration of cortical development and abnormal GABAergic neurotransmission at the impactful time when intracortical circuits form.

Keywords

Cortical Development; GABA; GABA-Mediated Synaptic Activity; BrdU; Reelin

Exposure to ethanol in utero may result in fetal alcohol spectrum disorder (FASD), hallmarked by varying degrees of cognitive deficits, many of which are associated with abnormalities in processing within the cortical circuitry (Sadrian et al., 2013; Shawa et al., 2013). Developmentally, the formation of cortical circuits is a tightly regulated sequel of events involving genesis, migration, and integration of inhibitory and excitatory neurons.

Cajal–Retzius cells, as the transient and first-born neurons of the embryonic rodent cortex, arise from pallial and subpallial origins and migrate tangentially below the pial surface to cover the entire neocortex (Bielle et al., 2005; García-Moreno et al., 2007). They release the glycoprotein reelin (D'Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995), implicated in regulating neuronal migration, layering, and neurogenesis in the developing cortex (Franco et al., 2011; Frotscher, 1998; Gil-Sanz et al., 2013; Gupta et al., 2003; Jossin and Cooper, 2011; Lakomá et al., 2011; Olson et al., 2006; Sekine et al., 2011). Thus, Cajal–Retzius cells are in a favorable position to orchestrate early aspects of corticogenesis, cortical patterning, and the establishment of nascent cortical circuits (Schwartz et al., 1998; Soriano and Del Río, 2005).

While the importance of Cajal–Retzius cells in corticogenesis is well-documented (Supèr et al., 2000), whether they are susceptible to ethanol exposure in utero is unexplored. In this study, pregnant mice were administered ethanol in a liquid diet that yielded moderate levels of blood alcohol ($x \bar{=} 20$ mg/dl). The adolescent offspring exposed in utero to ethanol displayed altered open-field activity, indicating a relevant neurobehavioral consequence of our in utero ethanol exposure paradigm. We started the maternal consumption regimen on embryonic day (E)9, the time of closure of the neural tube, to span the entire period of cortical development. In this light, beyond regulating migration and neuronal integration during corticogenesis, Cajal–Retzius cells have also been postulated to play a functional role within the developing cortical circuit (Aguiló et al., 1999; Radnikow et al., 2002; Soda et al., 2003). We show for the first time that they receive GABAergic synaptic input as early as E13.5 in the mouse neocortex. In addition, we report that ethanol exposure in utero early on in cortical development alters the genesis, distribution, and GABAergic synaptic input of Cajal–Retzius cells.

MATERIALS AND METHODS

Animals

All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Dartmouth Institutional Animal Care and Use Committee. This study employed the Ebf2-EGFP BAC transgenic mouse line, generated by the GENSAT project and obtained from Dr. Portera-Cailliau (Chowdhury et al., 2010; Gong et al., 2003). For time-pregnant mating, pairs of male and female mice were housed overnight, with the following day designated as E0.5. Embryos and postnatal pups of either sex were included in this study. The day of birth was designated as P0. The age range between P35 and P57 was operationally defined to be equivalent to the period of adolescence.

Maternal Ethanol Consumption

Our previous work employed a liquid diet regimen of moderate maternal ethanol consumption throughout gestation (Cuzon et al., 2008). In this study, we shortened the time frame of in utero ethanol exposure to target cortical development specifically, starting at E9.5 (Fig. 1A), when neurulation is complete and corticogenesis has just begun. Pregnant dams were individually housed and assigned to 1 of 2 groups: ethanol-fed or control-fed.

Mice were maintained under normal 12/12 hour light/dark cycle on a liquid diet (Research Diets, New Brunswick, NJ) supplemented with ethanol (2% w/w; ethanol-fed group) or an isocaloric control diet containing maltose (control-fed group); water was available ad libitum. The liquid food was replenished daily between 3:00 and 5:00 PM, when the amount consumed was measured and the dams weighed. Mice were maintained on their respective diets until parturition, after which they were returned to standard chow. Dam blood alcohol level (BAL; $\bar{x} = 20.2 \pm 3.25$ mg/dl) was assessed by blood collected via the tail vein at 11:30 PM on E15.5 utilizing an Analox Instruments GM7 series analyzer (Lunenburg, MA). Our model of maternal ethanol consumption did not affect litter size (control $\bar{x} = 9.67 \pm 1.1$ pups; EtOH $\bar{x} = 9.0 \pm 1.4$ pups; unpaired *t*-test, $p > 0.05$).

Electrophysiology

Neonatal (P2-P3) mice were euthanized by CO₂ asphyxiation, their brains were dissected and immersed in ice-cold oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 124; KCl 5.0; MgCl₂ 2.0; CaCl₂ 2.0; NaH₂PO₄ 1.25; NaHCO₃ 26; D-glucose 10 (pH = 7.4, adjusted with 1N NaOH). To prepare embryonic and early postnatal hemicortex whole-mounts, a transverse cut was made at the base of the telencephalic vesicle, the overlying pia was removed, and the developing hippocampus, medial neocortex, and striatum resected (Fig. 2B). The wholemounts were incubated in oxygenated aCSF at room temperature for at least 60 minutes before electrophysiological recording.

A hemicortex wholemount was transferred to a recording chamber mounted on a fixed-stage upright fluorescence microscope (BX51WI; Olympus, Melville, NY) equipped with Hoffman Modulation Contrast optics (Modulation Optics, Inc., Greenvale, NY), perfused with oxygenated aCSF (0.5 ml/min) and maintained at 32°C. Cajal-Retzius cells were identified by their location in the marginal zone or developing layer I, morphology, and emission of EGFP fluorescence. Borosilicate glass patch electrodes (4 to 6 MΩ in external solution) were filled with recording solution composed of (in mM): KCl 120; MgCl₂ 2.0; EGTA 1.0; HEPES 10; Mg²⁺ ATP 3.0 (pH = 7.3, adjusted with 1N KOH). Whole-cell recordings employed an AxoPatch 700B amplifier (Molecular Devices Inc., Sunnyvale, CA). Membrane currents were digitized online (Digidata 1320A; Molecular Devices Inc.) and recorded (Clampex v9.0; Molecular Devices Inc.) and analyzed offline using Clampfit v9.0 (Molecular Devices Inc.). To isolate GABAergic postsynaptic currents, CNQX (20 μM) and APV (20 μM) were included in the perfusion solution. In experiments that isolated miniature postsynaptic currents, 0.5 μM TTX was included in the bath to suppress action potential-driven synaptic activity. GABA reversal potential was determined under perforated patch conditions with patch electrodes filled with recording solution composed of (in mM): CsCl 130; HEPES 10; CaCl₂ 2; EGTA 11 (pH = 7.3, adjusted with 1N CsOH), and containing 0.5 μg/ml gramicidin (Sigma, St. Louis, MO). Focal GABA (20 μM) was applied via regulated pressure with the recorded cell clamped at varying membrane potentials.

Immunofluorescence: Imaging and Analysis

Time-pregnant dams were euthanized by CO₂ asphyxiation at 12 AM on E12.5, the embryos were quickly removed, their brains dissected, immerse-fixed in phosphate-buffered saline

(PBS) containing 4% paraformaldehyde (PFA)/0.1 M PBS, and cryoprotected in 30% sucrose/0.1 M PBS. Cryosections (30 μ m) were cut with a sliding microtome, mounted on glass slides, DAPI counter-stained, and cover slipped with FluorSave Reagent (Calbiochem, La Jolla, CA).

Postnatal mice were transcardially perfused with cold PBS followed by 4% PFA/0.1 M PBS. The brains were removed, immerse-fixed in 4% PFA/0.1 M PBS overnight at 4°C, and cryoprotected. Thirty-micron coronal cryosections were collected into PBS. For each animal, 10 sections per region were selected at equivalent rostral-caudal cortical levels (prefrontal or somatosensory) and used for counting EGFP-fluorescent Cajal–Retzius cells located in cortical layer I from the corticostriate juncture to the dorsal apex of the neocortex.

For 5-bromo-2-deoxyuridine (BrdU) pulse labeling, dams were injected with BrdU (100 mg/kg, intraperitoneally) at 12 _{AM} on E12.5. At 2 _{AM}, the embryo brains were removed, and 30- μ m cryosections were denatured in 2N HCl for 45 minutes, followed by 30 minutes in 0.1 M boric buffer (pH ~9.0). The sections were washed, blocked for 1 hour at room temperature in PBS/10% NGS/0.01% triton X-100, and incubated overnight at 4°C with mouse anti-BrdU (1:10 G3G4 hybridoma). The sections were then incubated overnight with Alexa Fluor 555 conjugated goat-anti-mouse secondary antibody (1:1,000; Invitrogen, Grand Island, NY). The sections were mounted, counterstained with DAPI, and coverslipped. Negative control with primary antibody omitted was processed in parallel. Reelin immunofluorescence staining was performed as above for BrdU, except with mouse antireelin primary antibody (1:1,000 G4; gift of Dr. André Goffinet).

Fluorescent images were captured digitally using a CCD camera (Hamamatsu, Hamamatsu City, Japan) fitted onto a spinning disk confocal microscope (BX61WI; Olympus) controlled by IPLab 4.0 software (BD Biosciences, San Jose, CA). The cortical hem was imaged in 10 sections from each embryo. The cortical hem was delineated as the region of interest, and a quantification vector defined by pixel intensity over background intensity was applied using IPLab 4.0 software to yield a percentage area of the cortical hem positive for fluorescence labeling (e.g., Fig. 4).

Open-Field Testing

Adolescent mice (P45-P50) were allowed to explore a novel moderately lit open-field environment (25 $\times$ 25 cm) for 30 minutes. The chamber (Coulbourn Instruments, Whitehall, PA) was equipped with infrared beams and sensors to detect activity and was connected to a computer to record data using TruScan software (Coulbourn Instruments).

Statistics

All histological analyses utilized the average region of interest data from ten 30- μ m tissue sections for each animal tested ($n = 1$: 1 animal = 10 sections). For comparisons of electrophysiological group data, n denotes the number of cells recorded. All groups consisted of data acquired from a minimum 3 individual animals from multiple litters. Group means were compared by unpaired t -test or 2-way analysis of variance (ANOVA) with Bonferroni post test as indicated, and reported as mean ($\bar{x}$) $\pm$ standard error.

Comparison of cumulative frequency histograms was made with the 2-sample Kolmogorov–Smirnov test and the distributions reported as median ($\tilde{x}$) and interquartile range (IQR).

RESULTS

Hyperactivity is a cardinal feature of FASD (Bhatara et al., 2006). We asked whether adolescent mice exposed in utero to ethanol displayed heightened spontaneous locomotor activity using an open-lid 25 × 25 cm clear plexiglass open-field arena with virtually demarcated activity zones (Fig. 1B). In Figure 1C–E, comparison between young adolescent (P45–P50) control mice (black dots) and those exposed to ethanol in utero (red dots) indeed revealed a hyperactive exploratory phenotype in the latter, hallmarked in the open-field paradigm by increased total distance traveled (Fig. 1C; control $\bar{x} = 4,910 \pm 279$ cm; EtOH $\bar{x} = 6,178 \pm 380$ cm), time spent in the center portion of the arena (Fig. 1D; control $\bar{x} = 335.8 \pm 30.4$ seconds; $\bar{x} = \text{EtOH } 476 \pm 52.9$ seconds), and number of entries into the center portion of the arena (Fig. 1E; control $\bar{x} = 159 \pm 11$ entries; EtOH $\bar{x} = 208.1 \pm 17$ entries). Thus, our maternal ethanol consumption paradigm contributed to the development of an enduring neurobehavioral phenotype in the ethanol-exposed offspring, reminiscent of the hyperactivity seen clinically in children with FASD (Ornoy and Ergaz, 2010; Steinhausen et al., 1993).

EGFP Labels Cajal–Retzius Cells Displaying Depolarizing GABAergic Current Responses in Embryonic Ebf2-EGFP Cortex

We confirmed the identity of the EGFP-fluorescent (EGFP+) cells in the cortex of embryonic Ebf2-EGFP mice (Fig. 2). Figure 2A illustrates images taken from the same DAPI-counterstained coronal section from an E12.5 Ebf2-EGFP cortex, showing EGFP profiles (Fig. 2A1) and reelin immunoreactivity (Fig. 2A2). Both EGFP fluorescence and reelin immunofluorescence were evident as a dense band along the pial aspect of the marginal zone along the entire extent of the embryonic neocortex. The merged, DAPI-counterstained image in Fig. 2A3 illustrates that the 2 patterns of fluorescence overlap. The hemicortex wholemounts were prepared from Ebf2-EGFP mice by making a transverse cut at the base of the telencephalic vesicle (Fig. 2B1), leaving the thalamus and the associated subpallial region (shaded area Fig. 2B2) and isolating the telencephalic vesicle (Fig. 2B3). The overlying pia was then removed, and the developing hippocampus, medial neocortex, and striatum were resected as outlined in Fig. 2B3, yielding an isolated neocortex with an average thickness of approximately 500 μm .

Figure 2C illustrates the top-down view of an E13.5 Ebf2-EGFP hemicortex wholemount. In Fig. 2C1, EGFP+ cells can be seen distributed in the marginal zone. Figure 2C2, taken from the same microscopic field, illustrates reelin-immunofluorescent cells with the same pattern of distribution. This is confirmed in Fig. 2C3, which demonstrates colocalization of EGFP and reelin immunofluorescence in cells that often displayed oval soma that emitted long processes extending parallel to the pial surface. The expression of reelin and the unique morphology provided neuroanatomical confirmation that the EGFP+ cells in the marginal zone are Cajal–Retzius cells (D'Arcangelo et al., 1995; Hirotsune et al., 1995; Meyer et al., 1999; Ogawa et al., 1995).

Whole-cell patch clamp recording of Cajal–Retzius cells in embryonic hemicortical wholemounts (Fig. 3A) revealed low levels of synaptic activity as early as E13.5 (Fig. 3B) that was reversibly blocked by bicuculline (20 μ M). Importantly, perforated patch recordings revealed that the GABA reversal potential, estimated by monitoring the polarity of GABA (20 μ M)-activated current responses at varying holding potentials (Fig. 3C), was more depolarized than the resting membrane potential (Fig. 3D; 5/5 cases; E_{GABA} $\bar{x}$ -34 ± 4 mV). Thus, the GABA-mediated synaptic activity encountered at E13.5 was depolarizing. This is consistent with and extends earlier studies (Mienville, 1998) reporting GABA-mediated depolarizing responses in Cajal–Retzius cells in the E18 rat neocortex.

Ethanol Exposure In Utero Increases the Proliferation of Cajal–Retzius Cells in the Cortical Hem

GABA_A receptor-mediated signaling regulates neurogenesis during embryonic cortical development (Haydar et al., 2000; LoTurco et al., 1995). We asked whether ethanol, as an allosteric modulator of the GABA_A receptor, alters the rate of neurogenesis in the cortical hem that produces the majority of neocortical Cajal–Retzius cells (Meyer, 2010). We first standardized the criteria for defining the cortical hem in DAPI-counterstained sections (Fig. 4A, B), and then quantified the percentage area of the region of interest occupied by the EGFP⁺ cells. Comparative analysis of EGFP fluorescence at E12.5 in the control vis-à-vis the ethanol-exposed groups indicated that the area of cortical hem populated by EGFP⁺ cells was significantly greater in embryos exposed to ethanol in utero (Fig. 4C; control $\bar{x}$ $9.5 \pm 1.7\%$; EtOH $\bar{x}$ $19.7 \pm 0.7\%$).

The above results prompted an experiment to examine whether increased proliferation might be a contributing factor. Pregnant dams were placed on an ethanol-containing or control liquid diet starting on E9.5, intraperitoneally injected with BrdU on E12.5 at 12 AM when the BAL typically peaked ($\bar{x}$ 20.2 ± 3.25 mg/dl), and sacrificed 2 hours thereafter. Figure 4D illustrates the extent of cellular BrdU labeling in the E12.5 forebrain. The percentage of the total area outlined as cortical hem occupied by cells emitting BrdU immunofluorescence (Fig. 4E) was greater in the ethanol-exposed embryos ($\bar{x}$ $37.4 \pm 3.1\%$) than control embryos ($\bar{x}$ $15.3 \pm 1.2\%$) (Fig. 4F). This difference in the dispersion of EGFP⁺ and BrdU⁺ immunofluorescent cells was not accompanied by any difference between groups in the cortical hem (control $\bar{x}$ $32,230 \pm 883$ μm^2 , $n = 9$; EtOH $\bar{x}$ $31,410 \pm 1,464$ μm^2 , $n = 11$; unpaired t -test $p > 0.05$).

Ethanol Exposure In Utero Increases the Density of Cajal–Retzius Cells in the Neonatal Cortex

We asked whether the observed increase in the production of Cajal–Retzius cells in the embryonic cortex persists as an increase in their number later in postnatal development. Figure 5A illustrates that the dense band of EGFP⁺ Cajal–Retzius cells observed in the embryonic cortex persists in the marginal zone (or emerging cortical layer I) of P0 Ebf2-EGFP mouse cortex (top panel); immunostaining with the reelin antibody revealed an identical distribution pattern (middle panel) that overlapped with that of Ebf2-EGFP fluorescence (bottom panel). Consistent with increased proliferation and Ebf2 expression in the embryonic cortical hem, we found more EGFP⁺ Cajal–Retzius cells in layer I of the

prefrontal cortex of P0 (control $\bar{x} = 16.27 \pm 0.49$ cells, $n = 4$; EtOH $\bar{x} = 23.43 \pm 0.32$ cells, $n = 4$) and P2 (control $\bar{x} = 10.8 \pm 0.62$ cells, $n = 4$; EtOH $\bar{x} = 16.55 \pm 1.1$ cells, $n = 6$) Ebf2-EGFP mice exposed to ethanol in utero (Fig. 5B). This ethanol-induced effect was not limited to the prefrontal cortex, as a similar increase was also found in the neonatal somatosensory cortex (Fig. 5C) both at P0 (control $\bar{x} = 16.14 \pm 0.24$ cells, $n = 4$; EtOH $\bar{x} = 24.7 \pm 0.67$ cells, $n = 5$) and at P2 (control $\bar{x} = 13.02 \pm 0.25$ cells, $n = 3$; EtOH $\bar{x} = 19.44 \pm 0.85$ cells, $n = 5$). The effect was transient, insofar as it was reduced to control levels by P4 in both prefrontal (control $\bar{x} = 8.19 \pm 0.49$ cells, $n = 6$; EtOH $\bar{x} = 10.07 \pm 0.33$ cells, $n = 4$) and somatosensory (control $\bar{x} = 11.74 \pm 0.43$ cells, $n = 3$; EtOH $\bar{x} = 12.53 \pm 0.11$ cells, $n = 3$) cortices.

Ethanol Exposure In Utero Enhances GABAergic Synaptic Input to Cajal–Retzius Cells

Cajal–Retzius cells participate in the nascent cortical circuit, receiving GABAergic input from multiple sources, notably GABAergic subplate neurons (Myakhar et al., 2011). The representative traces in Fig. 6A, B illustrate spontaneous GABA-mediated synaptic currents recorded in P2–P3 hemi-cortex wholemounts of control (Fig. 6A) and ethanol-exposed (Fig. 6B) offspring. The wholemount preparation preserved the developing intracortical circuitry and the dendrites of Cajal–Retzius cells that ran parallel to the pial surface since, under control conditions (Fig. 6A), action potential-dependent overriding GABA-mediated postsynaptic events clustered to form burst-type barrages that were blocked in the presence of 0.5 μ M TTX (Fig. 7A). The cumulative amplitude distribution histogram of GABA-mediated synaptic events in Fig. 6C shows a rightward shift from control (black line) with ethanol exposure (red line), indicating a significant increase in the probability of occurrence of larger amplitude GABA-mediated synaptic events (control $\bar{x} = 61.04$ pA, IQR = 102.8 pA, $n = 4,319$ events; EtOH $\bar{x} = 86.06$ pA, IQR = 116.12 pA, $n = 2,430$ events). This increase in amplitude was also evident in the scatter plot of the mean amplitude of GABA-mediated events in individual cells (Fig. 6C, inset; control $\bar{x} = 59 \pm 6.1$ pA; EtOH $\bar{x} = 95.8 \pm 9.9$ pA). On the other hand, a rightward shift of the inter-event interval cumulative frequency distribution histogram (Fig. 6D) indicates fewer events occurring at short-duration inter-event intervals, suggesting decreased frequency (control $\bar{x} = 748.8$ ms, IQR = 2,023.5 ms, $n = 4,294$ events; EtOH $\bar{x} = 1,194.25$ ms, IQR = 2,923.5 ms, $n = 2,412$ events). However, this was not corroborated when data were analyzed on a per cell basis, as the presumed decrease in mean frequency turned out not to be statistically significant between the control and the ethanol-exposed groups (Fig. 6D inset; control $\bar{x} = 0.297 \pm 0.09$ Hz; EtOH $\bar{x} = 0.229 \pm 0.07$ Hz). Further analysis revealed that this apparent discrepancy could be accounted for by a greater probability of encountering spontaneous GABA-mediated synaptic activity with burst-like patterns (Fig. 6A) in control cells (12/24 cells) compared to ethanol-exposed cells (3/18 cells; Fisher's exact test $p < 0.05$). Additionally, the proportion of burst-like overriding GABA-mediated synaptic events was higher in control (380/4,319 events) than ethanol-exposed (55/2,430 events) cells (chi-square $p < 0.0001$). Thus, rather than affecting frequency, ethanol exposure in utero altered the pattern of spontaneous GABA-mediated synaptic input to Cajal–Retzius cells. Taken together with the observed increase in amplitude of spontaneous GABA-mediated synaptic events, the results suggested that in utero ethanol exposure acted postsynaptically to modify GABA_A receptor function, leading to a net enhancement of GABAergic synaptic transmission. In terms of passive

membrane properties of neonatal Cajal–Retzius cells, in utero ethanol exposure did not affect whole-cell capacitance (control $\bar{x} = 13.06 \pm 0.83$ pF, $n = 34$ cells; EtOH $\bar{x} = 13.62 \pm 1.11$ pF, $n = 31$ cells; unpaired t -test $p > 0.05$), input resistance (control $\bar{x} = 1.10 \pm 0.13$ G Ω , $n = 11$ cells; EtOH $\bar{x} = 0.91 \pm 0.1$ G Ω , $n = 11$ cells; unpaired t -test $p > 0.05$), or resting membrane potential (control $\bar{x} = -47.5 \pm 4.5$ mV, $n = 11$ cells; EtOH $\bar{x} = -39.3 \pm 3$ mV, $n = 11$ cells; unpaired t -test $p > 0.05$).

To assess further the in utero ethanol-induced alterations in GABA-mediated synaptic activity, we monitored miniature GABA-mediated synaptic activities in control (Fig. 7A) and ethanol-exposed (Fig. 7B) Cajal–Retzius cells in the presence of 0.5 μ M TTX (Otis and Mody, 1992). Consistent with the increase in the amplitude of spontaneous GABA-mediated synaptic events, Fig. 7C illustrates that the population-based cumulative amplitude distribution histogram obtained from ethanol-exposed Cajal–Retzius cells shifted rightward (control $\bar{x} = 45.8$ pA, IQR = 31.1 pA, $n = 859$ events; EtOH $\bar{x} = 58$ pA, IQR = 61 pA, $n = 1,224$ events), and this was corroborated by a statistically significant increase in the amplitude of the mean miniature GABAergic events per cell between control ($\bar{x} = 53.15 \pm 2.78$ pA) and ethanol-exposed ($\bar{x} = 80.24 \pm 5.21$ pA) groups (Fig. 7C, inset). Mono-exponential fit of scaled average events revealed significantly faster decay kinetics of miniature GABA-mediated events from ethanol-exposed cells, as reflected in a decreased decay time constant (control $\bar{x} = 22.02 \pm 2.14$ ms, $n = 17$ cells; EtOH $\bar{x} = 15.8 \pm 0.79$, $n = 13$ cells; unpaired t -test $p < 0.05$) without a change in the 20 to 80% rise time (control $\bar{x} = 0.79 \pm 0.1$ ms, $n = 17$ cells; EtOH $\bar{x} = 0.55 \pm 0.1$ ms, $n = 13$ cells; unpaired t -test $p > 0.05$). Importantly, the miniature GABA-mediated synaptic events occurred at a higher frequency in ethanol-exposed Cajal–Retzius cells, as reflected by a leftward shift in the cumulative frequency distribution histogram (Fig. 7D; control $\bar{x} = 4,919$ ms, IQR = 11,146.8 ms, $n = 842$ events; EtOH $\bar{x} = 2,931.9$ ms, IQR = 6,965.8 ms, $n = 1,210$ events), and a commensurate increase in mean frequency analyzed on a per cell basis (Fig. 7D, inset; control $\bar{x} = 0.084 \pm 0.016$ Hz; EtOH $\bar{x} = 0.146 \pm 0.02$ Hz). Overall, these results indicate that ethanol exposure in utero augments the peak amplitude and frequency of miniature GABA-mediated synaptic events through both a postsynaptic alteration of GABA_A receptor function as well as a presynaptic enhancement of transmitter release.

DISCUSSION

Aberrant cortical form and function contribute to disrupted behaviors in FASD, including hyperactivity (Bhatara et al., 2006). Atypical cortical function in FASD may be due to improper GABA-dependent establishment of inhibitory/excitatory balance within cortical circuits (Sadrian et al., 2013; Shawa et al., 2013). Prominent among the myriad intrinsic and extrinsic factors that contribute to establishing proper cortical form and function are the reelin-secreting Cajal–Retzius cells. They orchestrate cortico-genetic events such as proliferation, migration, and positioning of immature cortical neurons, the interplay of which culminate in the well-documented developmental role of Cajal–Retzius cells and reelin in staging the tightly coordinated inside-out patterning and layering of the cortex (D'Arcangelo et al., 1995; Franco et al., 2011; Frotscher, 1998; Gil-Sanz et al., 2013; Gupta et al., 2003; Hirotsune et al., 1995; Jossin and Cooper, 2011; Lakom a et al., 2011; Ogawa et al., 1995; Olson et al., 2006; Sekine et al., 2011; Supèr et al., 2000).

This study is the first to investigate the effects of in utero exposure to a moderate level of ethanol (20 mg/dl) on the disposition of Cajal–Retzius cells in the embryonic and neonatal cortex. The Ebf2-EGFP transgenic mouse line facilitated our visualization and identification of Cajal–Retzius cells. In addition, we modified our previously established chronic maternal ethanol consumption paradigm (Cuzon et al., 2008) to begin on E9.5 to coincide temporally with the onset of corticogenesis. The major findings are that exposure to ethanol in utero: (i) escalates the genesis and expands the distribution of Cajal–Retzius cells in the developing cortex; and (ii) alters the pattern of GABA-mediated synaptic activity and raises the overall GABAergic tone on neonatal Cajal–Retzius cells.

In Utero Ethanol Exposure Increases Cajal–Retzius Cell Genesis and Expands Their Distribution in the Developing Cortex

We hypothesized that in utero ethanol exposure would alter Cajal–Retzius cell genesis and distribution within the cortex, because enhanced neurogenesis has been reported in other brain regions in response to moderate doses of ethanol exposure in utero (Chang et al., 2012). Indeed, we found increased BrdU activity in the cortical hem at E12.5, when this region is almost exclusively neurogenic (Anthony and Heintz, 2008; Götz and Huttner, 2005). As the cortical hem gives rise to a large contingent of Cajal–Retzius cells, this suggests that in utero ethanol exposure increased their rate of genesis. This, in turn, accounts for the expanded spread of the EGFP+ Cajal–Retzius cells observed within the cortical hem. Taken together, our results indicate that exposure to ethanol in utero enhances the proliferation of Cajal–Retzius cells.

Beyond embryonic development, the enhanced proliferative activity manifested as a transient increase in the density of layer I Cajal–Retzius cells in the cortices of P0–P4 neonates exposed to ethanol in utero. The basis of this transient increase remains to be elucidated. One might postulate that given the decrease in the number and density of Cajal–Retzius cells as development progresses, programmed apoptotic cell death during the first postnatal week in the rodent cortex might be responsible for the demise of supernumerary cortical neurons, including the Cajal–Retzius cells (Chowdhury et al., 2010; Derer and Derer, 1990). On the other hand, if one were to take the view that cell death does not account for the fate of Cajal–Retzius cells (Marín-Padilla, 1990; Parnavelas and Edmunds, 1983), then it may be that the rapid expansion of the cortex during the first neonatal week lowers their density to a degree that cell counts alone could not resolve any difference between the control and in utero ethanol-exposed cortices. In either case, it is conceivable that the changes in the distribution of Cajal–Retzius cells observed in the prefrontal and somatosensory cortices following ethanol exposure in utero, albeit temporary, may have affected indelibly the ability of Cajal–Retzius cells to play out their spatiotemporally dependent roles in the developing cortex. This notion needs experimental testing in future studies.

In Utero Ethanol Exposure Alters GABA-Mediated Synaptic Activity and Raises GABAergic Tone in Neonatal Cajal–Retzius Cells

Our results indicate that Cajal–Retzius cells receive GABA_A receptor-mediated synaptic input as early as E13.5. Thus, these synapses are vulnerable to the teratogenic influences of

ethanol early on in corticogenesis. Cajal–Retzius cells contribute to the generation and maintenance of correlated activity of the developing neocortex, which is required for proper development of the intracortical circuit (Aguiló et al., 1999; Schwartz et al., 1998; Soda et al., 2003). Their response to GABA_A receptor activation is almost exclusively depolarizing (Mienville, 1998; Pozas et al., 2008), and alteration in the strength or patterning of the GABAergic synaptic input may functionally skew developing circuits (Huang, 2009; Wang and Kriegstein, 2008).

We found in Cajal–Retzius cells a significant decrease in the occurrence of GABA-mediated synaptic events with short inter-event intervals in cortices exposed in utero to ethanol. However, this difference was not apparent when the average frequency of spontaneous GABAergic events was analyzed between groups on a per cell basis. The apparent discrepancy between analysis at the population and individual cell levels suggests that a change in the patterning of spontaneous GABAergic synaptic events may have occurred. Indeed, while “burst-like” spontaneous GABAergic synaptic events were regularly observed under control conditions, they were rarely encountered in ethanol-exposed Cajal–Retzius cells. Importantly, TTX abolished the burst-like synaptic activity, indicating its dependence on action potential-driven input. Our data indicate that the increase in spontaneous GABAergic inter-event interval consequent to in utero ethanol exposure is likely due to a change in the pattern of action potential-driven GABA-mediated synaptic activity.

Consistent with reports of chronic ethanol administration increasing the probability of release at GABAergic terminals (Roberto et al., 2006; Siggins et al., 2005), our results favored the occurrence of shorter inter-event intervals between miniature GABA-mediated synaptic events in Cajal–Retzius cells from in utero ethanol-exposed cortices. This, coupled with the increase in the frequency of miniature events, suggests enhanced probability of vesicular GABA release from presynaptic sites, more GABAergic synapses per Cajal–Retzius cell, or both. Postsynaptically, our analyses pointed to increased amplitude of both spontaneous and miniature GABA-mediated events in ethanol-exposed Cajal–Retzius cells. Kinetic analysis of miniature GABA-mediated events from control and ethanol-exposed cells revealed a change in decay rate, suggestive of a change in postsynaptic GABA_A receptor subunit composition. Indeed, brain region-specific changes in GABA_A receptor subunit composition occur following both acute and chronic exposure to ethanol, with faster miniature event decay kinetics being a common physiological outcome (Liang et al., 2007; Lindemeyer et al., 2014; Matthews et al., 1998). Overall, we propose that, with ethanol exposure in utero, these pre- and postsynaptic considerations converge to result in a net increase in GABAergic tone, exacerbating the depolarizing influence of GABA-mediated synaptic input to neonatal Cajal–Retzius cells.

The results presented here provide evidence that Cajal–Retzius cells are susceptible to a moderate-dose ethanol exposure in utero administered via maternal consumption. Given the importance of Cajal–Retzius cells and their activity-dependent participation in orchestrating proper cortico-genesis, the findings in this study inform future investigations in testing the hypothesis that changes in Cajal–Retzius cell disposition underlie in part the long-term adverse consequences of in utero ethanol exposure on cortical form and function in FASD.

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REFERENCES

- Aguilo A, Schwartz TH, Kumar VS, Peterlin ZA, Tsiola A, Soriano E, Yuste R. Involvement of Cajal–Retzius neurons in spontaneous correlated activity of embryonic and postnatal layer 1 from wild-type and reeler mice. *J Neurosci*. 1999; 19:10856–10868. [PubMed: 10594067]
- Anthony TE, Heintz N. Genetic lineage tracing defines distinct neurogenic and gliogenic stages of ventral telencephalic radial glial development. *Neural Dev*. 2008; 3:30. [PubMed: 18986511]
- Bhatara V, Loudenberg R, Ellis R. Association of attention deficit hyperactivity disorder and gestational alcohol exposure: an exploratory study. *J Atten Disord*. 2006; 9:515–522. [PubMed: 16481668]
- Bielle F, Griveau A, Narboux-Nême N, Vigneau S, Sigrist M, Arber S, Wassef M, Pierani A. Multiple origins of Cajal–Retzius cells at the borders of the developing pallium. *Nat Neurosci*. 2005; 8:1002–1012. [PubMed: 16041369]
- Chang G-Q, Karatayev O, Liang SC, Barson JR, Leibowitz SF. Prenatal ethanol exposure stimulates neurogenesis in hypothalamic and limbic peptide systems: possible mechanism for offspring ethanol overconsumption. *Neuroscience*. 2012; 222:417–428. [PubMed: 22742906]
- Chowdhury TG, Jimenez JC, Bomar JM, Cruz-Martin A, Cantle JP, Porter-a-Cailliau C. Fate of Cajal–Retzius neurons in the postnatal mouse neocortex. *Front Neuroanat*. 2010; 4:10. [PubMed: 20339484]
- Cuzon VC, Yeh PWL, Yanagawa Y, Obata K, Yeh HH. Ethanol consumption during early pregnancy alters the disposition of tangentially migrating GABAergic interneurons in the fetal cortex. *J Neurosci*. 2008; 28:1854–1864. [PubMed: 18287502]
- D'Arcangelo G, Miao GG, Chen SC, Soares HD, Morgan JI, Curran T. A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature*. 1995; 374:719–723. [PubMed: 7715726]
- Derer P, Derer M. Cajal–Retzius cell ontogenesis and death in mouse brain visualized with horseradish peroxidase and electron microscopy. *Neuroscience*. 1990; 36:839–856. [PubMed: 2234416]
- Franco SJ, Martinez-Garay I, Gil-Sanz C, Harkins-Perry SR, Müller U. Reelin regulates cadherin function via Dab1/Rap1 to control neuronal migration and lamination in the neocortex. *Neuron*. 2011; 69:482–497. [PubMed: 21315259]
- Frotscher M. Cajal–Retzius cells, reelin, and the formation of layers. *Curr Opin Neurobiol*. 1998; 8:570–575. [PubMed: 9811621]
- García-Moreno F, Lopez-Mascaraque L, De Carlos JA. Origins and migratory routes of murine Cajal–Retzius cells. *J Comp Neurol*. 2007; 500:419–432. [PubMed: 17120279]
- Gil-Sanz C, Franco SJ, Martinez-Garay I, Espinosa A, Harkins-Perry S, Müller U. Cajal–Retzius cells instruct neuronal migration by coincidence signaling between secreted and contact-dependent guidance cues. *Neuron*. 2013; 79:461–477. [PubMed: 23931996]
- Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, Nowak NJ, Joyner A, Leblanc G, Hatten ME, Heintz N. A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature*. 2003; 425:917–925. [PubMed: 14586460]
- Götz M, Huttner WB. The cell biology of neurogenesis. *Nat Rev Mol Cell Biol*. 2005; 6:777–788. [PubMed: 16314867]
- Gupta A, Sanada K, Miyamoto DT, Rovelstad S, Nadarajah B, Pearlman AL, Brunstrom J, Tsai L-H. Layering defect in p35 deficiency is linked to improper neuronal-glial interaction in radial migration. *Nat Neurosci*. 2003; 6:1284–1291. [PubMed: 14608361]
- Haydar TF, Wang F, Schwartz ML, Rakic P. Differential modulation of proliferation in the neocortical ventricular and subventricular zones. *J Neurosci*. 2000; 20:5764–5774. [PubMed: 10908617]

- Hirotsune S, Takahara T, Sasaki N, Hirose K, Yoshiki A, Ohashi T, Kusakabe M, Murakami Y, Muramatsu M, Watanabe S, Nakao K, Katsuki M, Hayashizaki Y. The reeler gene encodes a protein with an EGF-like motif expressed by pioneer neurons. *Nat Genet.* 1995; 10:77–83. [PubMed: 7647795]
- Huang ZJ. Activity-dependent development of inhibitory synapses and innervation pattern: role of GABA signalling and beyond. *J Physiol.* 2009; 587:1881–1888. [PubMed: 19188247]
- Jossin Y, Cooper JA. Reelin, Rap1 and N-cadherin orient the migration of multipolar neurons in the developing neocortex. *Nat Neurosci.* 2011; 14:697–703. [PubMed: 21516100]
- Lakomá J, Garcia-Alonso L, Luque JM. Reelin sets the pace of neo-cortical neurogenesis. *Development.* 2011; 138:5223–5234. [PubMed: 22069190]
- Liang J, Suryanarayanan A, Abriam A, Snyder B, Olsen RW, Spigelman I. Mechanisms of reversible GABAA receptor plasticity after ethanol intoxication. *J Neurosci.* 2007; 27:12367–12377. [PubMed: 17989301]
- Lindemeyer AK, Liang J, Marty VN, Meyer EM, Suryanarayanan A, Olsen RW, Spigelman I. Ethanol-induced plasticity of GABAA receptors in the basolateral amygdala. *Neurochem Res.* 2014; 39:1162–1170. [PubMed: 24710789]
- LoTurco JJ, Owens DF, Heath MJ, Davis MB, Kriegstein AR. GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. *Neuron.* 1995; 15:1287–1298. [PubMed: 8845153]
- Marín-Padilla M. Three-dimensional structural organization of layer I of the human cerebral cortex: a Golgi study. *J Comp Neurol.* 1990; 299:89–105. [PubMed: 2212113]
- Matthews DB, Devaud LL, Fritschy JM, Sieghart W, Morrow AL. Differential regulation of GABA(A) receptor gene expression by ethanol in the rat hippocampus versus cerebral cortex. *J Neurochem.* 1998; 70:1160–1166. [PubMed: 9489737]
- Meyer G. Building a human cortex: the evolutionary differentiation of Cajal–Retzius cells and the cortical hem. *J Anat.* 2010; 217:334–343. [PubMed: 20626498]
- Meyer G, Goffinet AM, Fair en A. Feature article: what is a Cajal–Retzius cell? A reassessment of a classical cell type based on recent observations in the developing neocortex. *Cereb Cortex.* 1999; 9:765–775. [PubMed: 10600995]
- Mienville J-M. Persistent depolarizing action of GABA in rat Cajal–Retzius cells. *J Physiol.* 1998; 512:809–817. [PubMed: 9769423]
- Myakhar O, Unichenko P, Kirischuk S. GABAergic projections from the subplate to Cajal–Retzius cells in the neocortex. *NeuroReport.* 2011; 22:525–529. [PubMed: 21666518]
- Ogawa M, Miyata T, Nakajima K, Yagyu K, Seike M, Ikenaka K, Yamamoto H, Mikoshiba K. The reeler gene-associated antigen on Cajal–Retzius neurons is a crucial molecule for laminar organization of cortical neurons. *Neuron.* 1995; 14:899–912. [PubMed: 7748558]
- Olson EC, Kim S, Walsh CA. Impaired neuronal positioning and dendritogenesis in the neocortex after cell-autonomous Dab1 suppression. *J Neurosci.* 2006; 26:1767–1775. [PubMed: 16467525]
- Ornoy A, Ergaz Z. Alcohol abuse in pregnant women: effects on the fetus and newborn, mode of action and maternal treatment. *Int J Environ Res Public Health.* 2010; 7:364–379. [PubMed: 20616979]
- Otis TS, Mody I. Modulation of decay kinetics and frequency of GABAA receptor-mediated spontaneous inhibitory postsynaptic currents in hippocampal neurons. *Neuroscience.* 1992; 49:13–32. [PubMed: 1357584]
- Parnavelas JG, Edmunds SM. Further evidence that Retzius–Cajal cells transform to nonpyramidal neurons in the developing rat visual cortex. *J Neurocytol.* 1983; 12:863–871. [PubMed: 6644358]
- Pozas E, Paco S, Soriano E, Aguado F. Cajal–Retzius cells fail to trigger the developmental expression of the Cl[−] extruding co-transporter KCC2. *Brain Res.* 2008; 1239:85–91. [PubMed: 18789906]
- Radnikow G, Feldmeyer D, Lübke J. Axonal projection, input and output synapses, and synaptic physiology of Cajal–Retzius cells in the developing rat neocortex. *J Neurosci.* 2002; 22:6908–6919. [PubMed: 12177189]
- Roberto M, Treistman SN, Pietrzykowski AZ, Weiner J, Galindo R, Mameli M, Valenzuela F, Zhu PJ, Lovinger D, Zhang TA, Hendricson AH, Morrisett R, Siggins GR. Actions of acute and chronic ethanol on pre-synaptic terminals. *Alcohol Clin Exp Res.* 2006; 30:222–232. [PubMed: 16441271]

- Sadrian B, Wilson DA, Saito M. Long-lasting neural circuit dys-function following developmental ethanol exposure. *Brain Sci.* 2013; 3:704–727. [PubMed: 24027632]
- Schwartz TH, Rabinowitz D, Unni V, Kumar VS, Smetters DK, Tsiola A, Yuste R. Networks of coactive neurons in developing layer 1. *Neuron.* 1998; 20:541–552. [PubMed: 9539127]
- Sekine K, Honda T, Kawauchi T, Kubo K, Nakajima K. The outermost region of the developing cortical plate is crucial for both the switch of the radial migration mode and the Dab1-dependent “inside-out” lamination in the neocortex. *J Neurosci.* 2011; 31:9426–9439. [PubMed: 21697392]
- Shaw HE, Abbott CW, Huffman KJ. Prenatal ethanol exposure disrupts intraneocortical circuitry, cortical gene expression, and behavior in a mouse model of FASD. *J Neurosci.* 2013; 33:18893–18905. [PubMed: 24285895]
- Siggins GR, Roberto M, Nie Z. The tippy terminal: presynaptic effects of ethanol. *Pharmacol Ther.* 2005; 107:80–98. [PubMed: 15963352]
- Soda T, Nakashima R, Watanabe D, Nakajima K, Pastan I, Nakanishi S. Segregation and coactivation of developing neocortical layer 1 neurons. *J Neurosci.* 2003; 23:6272–6279. [PubMed: 12867512]
- Soriano E, Del Río JA. The cells of Cajal–Retzius: still a mystery one century after. *Neuron.* 2005; 46:389–394. [PubMed: 15882637]
- Steinhausen HC, Willms J, Spohr HL. Long-term psychopathological and cognitive outcome of children with fetal alcohol syndrome. *J Am Acad Child Adolesc Psychiatry.* 1993; 32:990–994. [PubMed: 8407775]
- Supér H, Del Río JA, Martínez A, Pérez-Sust P, Soriano E. Disruption of neuronal migration and radial glia in the developing cerebral cortex following ablation of Cajal–Retzius cells. *Cereb Cortex.* 2000; 10:602–613. [PubMed: 10859138]
- Wang DD, Kriegstein AR. GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation. *J Neurosci.* 2008; 28:5547–5558. [PubMed: 18495889]

Fig. 1.

Offspring exposed to ethanol in utero throughout embryonic corticogenesis are hyperactive at P30. **(A)** Experimental timeline of in utero ethanol exposure and experimental end points. Time-pregnant dams were fed a liquid diet without (control) or with 2% ethanol w/w (EtOH) from E9.5 until birth (time frame indicated by red bar); Cajal–Retzius cell genesis was assessed at E12.5 (1); counts of neonatal Cajal–Retzius cells were determined between P0 and P4 (2); electrophysiology was performed at P2–P3 (3); open-field behavioral testing was conducted between P45 and P50 (4). **(B)** Layout of open-field arena; the computer-based software defines the area labeled black as the center, and the surrounding gray area as the margin. **(C)** Distance traveled by adolescent offspring, each given 30 minutes to explore the 25 × 25 cm open-field arena. **(D)** Time spent in center of the arena. **(E)** Number of transitions from margin to center of the arena. Unpaired *t*-test, **p* < 0.05. Each dot represents data from an individual mouse derived from 2 control-fed and 2 ethanol-fed dams.

Fig. 2. EGFP in the embryonic cortex of the Ebf2-EGFP mouse labels Cajal–Retzius cells (**A**) E12.5 Ebf2-EGFP coronal section displaying superficial EGFP fluorescence in the cortical hem and preplate (A1), reelin immunofluorescence (A2), and merged image with DAPI counterstaining (A3); scale bar = 100 μ m. (**B**) Diagram illustrating the preparation of hemicortex wholemount. Dissection along the dotted red line (B1) leaves behind the thalamus and associated subpallial region (shaded area B2), isolating the telencephalic vesicle (B3). The dotted lines in B3 demarcate the dissection borders for removal of the hippocampus (green), striatum (orange), ventral telencephalon and olfactory bulb (red), as well as the medial aspect of the neocortex (blue). This sequence of dissections yields an isolated wholemount preparation of the neocortex (B4). A top-down view of E13.5 Ebf2-EGFP hemicortex wholemount processed to reveal the distribution of EGFP-expressing (C1) and reelin-immunofluorescent Cajal–Retzius cells (C2); the merged image (C3) confirms reelin expression in all visible EGFP-expressing cells; scale bar = 25 μ m.

Fig. 3.

EGFP+ Cajal-Retzius in the embryonic cortex receive depolarizing GABAergic input. **(A)** An EGFP+ Cajal-Retzius cell being recorded with a multibarrel drug pipet assembly in the vicinity; scale bar = 10 μm. **(B)** Whole-cell recording of GABAergic postsynaptic currents from an EGFP+ Cajal-Retzius cell in the marginal zone of a E13.5 Ebf2-EGFP mouse; scale = 20 pA × 50 ms. **(C)** E13.5 Cajal-Retzius cell response to exogenously applied GABA (20 μM) at 3 holding potentials under perforated patch clamp conditions; scale = 50 pA × 1 s. Traces low-pass-filtered at 500 Hz. **(D)** The GABA reversal potential of an E13.5 Cajal-Retzius cell is depolarized relative its resting membrane potential (RMP).

Fig. 4.

Ethanol exposure in utero increases neurogenesis in the cortical hem. **(A)** Coronal section of E12.5 Ebf2-EGFP brain counterstained with DAPI; dotted white line outlines the cortical hem, scale bar = 100 μ m. **(B)** Representative isolated cortical hem regions of interest from control (top) and ethanol-exposed (bottom) embryos, scale bar = 100 μ m. **(C)** Quantification of percent Ebf2-EGFP cortical hem area positive for EGFP fluorescence. Each dot represents 1 embryo derived from 2 control-fed and 2 ethanol-fed dams. **(D)** E12.5 coronal brain section processed for BrdU immunofluorescence and counterstained with DAPI. Dotted white line outlines the cortical hem; scale bar = 100 μ m. **(E)** Isolated cortical hem regions of interest from control (top) and ethanol-exposed (bottom) embryos; scale bar = 100 μ m. **(F)** Quantification of percent cortical hem area positive for BrdU immunofluorescence. Each dot represents 1 embryo, derived from 2 control-fed and 2 ethanol-fed dams unpaired *t*-test, ****p* < 0.001.

Fig. 5.

In utero ethanol exposure increases Cajal–Retzius cells in the neonatal cortex. **(A)** Coronal section of P0 Ebf2-EGFP cortex depicting in the marginal zone (MZ) the location of EGFP+ cells (upper panel), reelin immunoreactive cells (middle panel), and their colocalization (lower panel); scale bar = 100 μm . Upper dotted white line delineates the pial surface; lower dotted white line marks the boundary of the cortical plate. **(B, C)** Number of EGFP+ Cajal–Retzius cells in the marginal zone/layer I of control and ethanol-exposed offspring in prefrontal (PFC; B) and somatosensory (SSC; C) cortices. Two-way ANOVA with Bonferroni post test, **** $p < 0.0001$. The number of animals used per age (n) equals the number of dams used per age.

Fig. 6.

Ethanol exposure in utero enhances the amplitude and alters the pattern of spontaneous GABAergic synaptic activity in Cajal–Retzius cells. (**A**, **B**) Spontaneous GABAergic synaptic events in P3 EGFP+ Cajal–Retzius cell from control (**A**) and in utero ethanol-exposed (**B**) off-spring. Scale = 50pA×20s. Note the burst-like activity in control recordings (black lines below trace and shown amplified below the bar with arrowheads marking overriding spontaneous GABA-mediated synaptic currents). Scale = 50pA×500ms. (**C**) Cumulative amplitude distribution of spontaneous GABA-mediated synaptic events from control (black line) and in utero ethanol-exposed (red line) neonates. The inset illustrates the mean amplitude of GABA-mediated events (control = black dot; EtOH = red dot); each dot

represents data from 1 Cajal–Retzius cell. **(D)** Cumulative inter-event interval distribution of spontaneous GABA-mediated synaptic events from control (black line) and in utero ethanol-exposed (red line) neonates. The inset illustrates the mean frequency of GABA-mediated events (control = black dot; EtOH = red dot); each dot represents data from 1 Cajal–Retzius cell. Two-sample Kolmogorov–Smirnov test, *** $p < 0.001$. Unpaired t -test, ** $p < 0.01$. Cells were recorded from 3 control and 3 ethanol-exposed neonates derived from 3 control-fed and 3 ethanol-fed dams.

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Fig. 7.

Ethanol exposure in utero enhances the amplitude and frequency of miniature GABAergic synaptic input to Cajal–Retzius cells. **(A, B)** Spontaneous miniature GABAergic synaptic events in P3 EFGP+ Cajal–Retzius cells from control **(A)** and in utero ethanol-exposed **(B)** off-spring. Scale = 50 pA × 10 s. **(C)** Cumulative amplitude distribution of miniature GABAergic synaptic events under control (black line) and in utero ethanol-exposed (red line) conditions. The inset illustrates the mean amplitude of GABA-mediated events (control = black dot; EtOH = red dot); each dot represents data from 1 Cajal–Retzius cell. **(D)** Cumulative inter-event interval distribution of miniature GABAergic synaptic events under control (black line) and in utero ethanol-exposed (red line) conditions. The inset illustrates the mean frequency of GABA-mediated events (control = black dot; EtOH = red dot); each dot represents data from 1 Cajal–Retzius cell. Two-sample Kolmogorov–Smirnov test,

**** $p < 0.0001$. Unpaired t -test, * $p < 0.05$, **** $p < 0.0001$. Cells were recorded from 3 control and 3 ethanol-exposed neonates derived from 3 control-fed and 3 ethanol-fed dams.

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