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Moderate prenatal alcohol exposure alters the number and function of GABAergic interneurons in the murine orbitofrontal cortex

Johnny A. Kenton¹, Tiahna Ontiveros¹, Clark W. Bird¹, Fernando C. Valenzuela^{1,2}, Jonathan L. Brigman^{1,2}

¹Department of Neurosciences, University of New Mexico School of Medicine, Albuquerque, NM 87131

²New Mexico Alcohol Research Center, University of New Mexico HSC, Albuquerque, NM 87131

Abstract

Exposure to alcohol during development produces Fetal Alcohol Spectrum Disorders (FASD), characterized by a wide range of effects that include deficits in multiple cognitive domains. Early identification and treatment of individuals with FASD remains a challenge because neurobehavioral alterations do not become a significant problem until late childhood and early adolescence. Understanding the mechanisms underlying low and moderate prenatal alcohol exposure (PAE) effects on behavior and cognition is essential for improved diagnosis and treatment. Here, we examined the functional and morphological changes in an area known to be involved in executive control, the orbitofrontal cortex (OFC). We found that a moderate PAE model, previously shown to impair behavioral flexibility and to alter OFC activity, *in vivo* produced moderate functional and morphological changes within the OFC of mice *in vitro*. Specifically, slice electrophysiological recordings of spontaneous inhibitory post-synaptic currents in OFC pyramidal neurons revealed a significant increase in the amplitude and area in PAE mice relative to controls. Immunohistochemistry uncovered an increase in calretinin-, but not somatostatin- or parvalbumin-expressing cortical interneurons in the OFC of PAE mice. Together these data suggest that moderate prenatal alcohol exposure alters the disinhibitory function in the OFC, which may contribute to the executive function deficits associated with FASD.

*Correspondence author: Jonathan L. Brigman, PhD, Department of Neurosciences, University of New Mexico School of Medicine, MSC08 4740, 1 University of New Mexico, Albuquerque, NM, USA 87131-0001, jbrigman@salud.unm.edu, Telephone: 505-272-2868, Fax: 505-272-8082.

Author Statement:

Johnny A. Kenton: Investigation, Methodology, Data curation, Formal analysis, Writing-Original draft preparation. **Tiahna Ontiveros:** Investigation. **Clark W. Bird:** Data curation, Validation, Visualization, Investigation. **C. Fernando Valenzuela:** Methodology, Validation Writing-Reviewing and Editing. **Jonathan L. Brigman:** Supervision, Conceptualization, Funding acquisition, Writing-Reviewing and Editing.

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Keywords

prenatal alcohol exposure; interneuron; electrophysiology; baseline; GABA; ethanol; development; cortex

1. Introduction

Even in the face of increasingly strong evidence that fetal alcohol exposure has long lasting negative effects on development, alcohol use by pregnant women remains pervasive (Ethen et al., 2009; Popova, Lange, Probst, Gmel, & Rehm, 2017). Human clinical research has demonstrated that even moderate levels of alcohol can have harmful effects on cognitive outcomes in children (Coles et al., 2015; Georgieff, Tran, & Carlson, 2018; Jarmasz, Basalah, Chudley, & Del Bigio, 2017). Studies in various animal models of prenatal alcohol exposure (PAE) have examined the effects that different alcohol doses and timings can have on the development and subsequent behavior of animals (reviewed in Marquardt & Brigman, 2016; Patten, Fontaine, & Christie, 2014). These wide ranging effects, termed Fetal Alcohol Spectrum Disorders (FASD), are often more subtle than those present in the more commonly discussed Fetal Alcohol Syndrome (FAS). While FAS is characterized by facial dysmorphology and learning and memory deficits, the alcohol-related neurodevelopmental disorders (ARND) are more difficult to detect in a timely manner, since affected individuals display typical early development with neurobehavioral problems appearing during early adolescence (Riley, Infante, & Warren, 2011). This feature of ARND, along with underreported drinking by pregnant mothers, makes it difficult both to identify and treat affected individuals, especially since key characteristics of ARND share similar features with other diagnoses such as attention-deficit hyperactivity disorder (Kingdon, Cardoso, & McGrath, 2016; Singer et al., 2017). Therefore, understanding the mechanisms underlying low and moderate PAE effects on behavior and cognition is essential for improved diagnosis and treatment.

Executive control is an umbrella term for those cognitive processes that are required for efficiently navigating a complex environment and includes working memory, attention and behavioral flexibility (Diamond, 2013). While studies have examined multiple domains of executive control across species, cognitive flexibility has become the hallmark measure of these processes due to its conserved circuitry and the availability of behavioral measures that translate well across species (Audet & Lefebvre, 2017). Deficiencies in behavioral flexibility (namely maladaptive perseveration, or an inability to stop previously advantageous behaviors) have been described in several neurodevelopmental and neuropsychiatric disorders, including schizophrenia, obsessive-compulsive disorder and FASD (Floresco, Zhang, & Enomoto, 2009; Gruner & Pittenger, 2017; Marquardt, Sigdel, Caldwell, & Brigman, 2014; Negron-Oyarzo, Aboitiz, & Fuentealba, 2016; Remijnse et al., 2013). Previous studies have provided strong evidence that frontal cortex, and more specifically the orbitofrontal cortex (OFC), is a necessary component for efficient reversal learning, making it a valuable measure of behavioral flexibility (D. A. Hamilton & Brigman, 2015; Stalnaker, Cooch, & Schoenbaum, 2015). We have previously shown that PAE negatively affects reversal learning on a touchscreen-based visual reversal learning task by increasing

maladaptive perseverative responding during the early stages of reversal (Marquardt et al., 2014) during which proper OFC functioning is critical (Brigman et al., 2013; Izquierdo, Brigman, Radke, Rudebeck, & Holmes, 2017). These deficits were accompanied by altered OFC firing and recruitment, and significant reduction in consistency of local field potentials (LFP) following choice behaviors (Marquardt, Cavanagh, & Brigman, 2020). The reversal stage of this task can be viewed as a shift from excitatory learning (initial discrimination) to more inhibitory learning (Izquierdo & Jentsch, 2012), which has been shown to be mediated by GABAergic interneurons (Bissonette, Bae, Suresh, Jaffe, & Powell, 2010). Studies in transgenic mouse lines displaying fewer interneurons have implicated inhibitory tone in the OFC as essential for efficient reversal behavior (Bissonette, Schoenbaum, Roesch, & Powell, 2015). Depending on the developmental timing of exposure, alcohol can produce differing outcomes, with prenatal exposure displaying increases in interneuron subtype numbers (Skorput & Yeh, 2016) and early postnatal exposure showing decreases (Smiley et al., 2015). These data suggest that disruption of interneuron function could be a mechanism by which PAE alters reversal learning performance on these visual learning tasks.

Cortical inhibitory interneurons act as a brake in the brain, modulating neuronal excitability through the release of γ -aminobutyric acid (GABA) (Ferguson & Gao, 2018; Tremblay, Lee, & Rudy, 2016). These cells are diverse in both form and action (Burkhalter, 2008; DeFelipe et al., 2013), differing not only in shape but also electrophysiological characteristics. The most common interneuron subtype, parvalbumin-positive (PV^+), has previously been shown to be crucial for learning and memory, particularly efficient reversal learning (Bissonette et al., 2015) and have properties that are distinguishable from other interneuron subtypes, such as somatostatin (SST^+) and calretinin (CR^+) (Casale, Foust, Bal, & McCormick, 2015). PAE has previously been shown to impact the migration and/or final number of interneurons in the cortex (Cuzon, Yeh, Yanagawa, Obata, & Yeh, 2008; G. F. Hamilton, Hernandez, Krebs, Bucko, & Rhodes, 2017; Saito et al., 2019; Shenoda, 2017), hippocampus (Bird, Taylor, Pinkowski, Chavez, & Valenzuela, 2018; Madden et al., 2020), and cerebellum (Nirgudkar, Taylor, Yanagawa, & Valenzuela, 2016). Additionally, binge-like ethanol exposure during the third trimester equivalent (blood alcohol concentration (BAC): ~100–300 mg/dL) decreases the number of fast-spiking interneurons in the adult mouse brain (Bird et al., 2018; Nirgudkar et al., 2016). Interestingly, one study examining the effects of low prenatal alcohol exposure levels (BAC: 25 mg/dL) in the fetal brain found an increase in fast-spiking interneuron numbers throughout the cortex (Cuzon et al., 2008), even though low level exposure has been shown to increase neuroapoptosis in the brains of mouse pups (Young & Olney, 2006). How moderate exposure to alcohol affects the number and properties of interneuron populations in the OFC specifically has yet to be addressed and could help explain the increased maladaptive perseveration observed in the reversal learning tasks.

Here, we utilized a previously established drinking-in-the-dark model (Akers et al., 2011; Brady, Allan, & Caldwell, 2012; Brady et al., 2013; Marquardt et al., 2014) to explore the effects that PAE had on populations of certain inhibitory interneuron populations in the mouse OFC. Utilizing immunohistochemistry, as well as *ex vivo* electrophysiology, we found that PAE mice demonstrated altered inhibitory post-synaptic currents in pyramidal neurons and an increased number of the CR^+ interneuron sub-type in female PAE mice. To

our knowledge, this is the first data to demonstrate alterations in interneurons in the mouse OFC after moderate prenatal exposure to alcohol.

2. Methods

All experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

2.1 Drinking-in-the-Dark Model of Prenatal Alcohol Exposure.

Female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in a limited access PAE paradigm as described previously (Brady et al., 2012; Brady et al., 2013; Marquardt et al., 2014). Briefly, 2 hours into the dark cycle, female mice were given access to either 0.066% (w/v) saccharin or an alcohol solution (5% w/v for 4 days, then 10% w/v) sweetened with 0.066% (w/v) saccharin for 4 hours per day (from 1000 to 1400 hrs). After 1 week of acclimation to the 10% alcohol or saccharin control solution, individual females were placed in a cage of a singly housed male for 2 hours immediately following the drinking period (~1400 hrs) for 5 days. Females continued to consume alcohol and saccharin solutions throughout the 5-day mating period. Pregnancy was positively determined by monitoring weight gain every 3 to 4 days. Access to alcohol was withdrawn beginning on postnatal day 0 using a step-down procedure over a 6-day period and offspring were weaned at ~23 days of age. The average alcohol consumption of the mothers of the offspring used in these studies was 4.17 ± 0.61 g alcohol/kg, in agreement with previous reports (Brady et al., 2012). This protocol produces BACs of ~80 mg/dL at the end of the 4-hour drinking period (Brady et al., 2012; Brady et al., 2013). Offspring of alcohol-drinking (PAE) and saccharin-drinking (SAC) mothers from successive breeding rounds were housed in groupings of 2 per cage in a temperature- and humidity-controlled vivarium under a reverse 12-hour light/dark cycle (lights off 0800 hrs).

2.2 Ex vivo electrophysiology.

Unless indicated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO). We used male C57BL/6J offspring mice (27.5 ± 0.4 g and 11–12 weeks of age) that were exposed to saccharin or alcohol during fetal development, as described above (1–2 cells/animal; 7 mice from 6 litters for SAC and 8 mice from 4 litters for PAE). We focused on male mice as a previous *in vivo* electrophysiological study did not detect sex-dependent differences on reversal correction errors during the perseverative phase of reversal learning (Marquardt ACER 2014) and the *in vivo* effects of PAE on OFC electrophysiology are better characterized in males (Marquardt 2020 Neuropharmacology). In addition, we used transgenic mice expressing the Venus fluorescence protein in GABAergic and glycinergic interneurons under the control of the vesicular GABA transporter (Venus-VGAT mice; at approximately 10 weeks) to assess differences in the electrophysiological properties of pyramidal neurons vs. interneurons (Y. Wang et al., 2009). Mice were deeply anesthetized with ketamine (250 mg/kg, i.p.) and transcardially perfused with 25 ml of ice-cold cutting solution containing (in mM): 220 sucrose, 26 NaHCO₃, 2 KCl, 1.3 NaH₂PO₄, 12 MgSO₄, 0.2 CaCl₂, 10 glucose, and 400 μ M ascorbic acid equilibrated with 95% O₂/5% CO₂. Brains

were carefully removed from the skull and immersed for 2 min into ice-cold cutting solution. Brains were then glued onto a petri dish, covered with ice-cold cutting solution, and sliced (300µm thick) in the coronal plane using a vibrating slicer (Vibratome 1000 plus, Leica Microsystems, Bannockburn, IL). Subsequently, slices were allowed to recover in artificial cerebral spinal fluid (ACSF) equilibrated with 95%O₂/5%CO₂ containing (in mM): NaCl, 125; KCl, 2; NaH₂PO₄, 1.3; NaCO₃, 26; glucose, 10, CaCl₂, 2; MgSO₄, 1; 400 µM ascorbic acid (300–310 mOsm, pH: 7.3–7.4) at 34–36°C for 40 minutes. Slices were then stored in the ACSF at 21–22°C, for at least 30 min prior to the start of electrophysiological recordings, in a holding chamber (model BSC-PC, Warner Instruments, Hamden, CT). To visualize the neurons, we used an Olympus BX50WI upright microscope (Olympus, Center Valley, PA) with infra-red differential interference contrast optics connected to a charge-coupled device camera (CCD100, DAGE-MTI, Michigan City, IN).

Recordings were performed from neurons located in deep layers of the lateral OFC, as previously described (Badanich, Mulholland, Beckley, Trantham-Davidson, & Woodward, 2013). Slices used for recordings were at approx. AP: +2.46. For all experiments, we used an internal solution containing (in mM): KCl, 135; HEPES, 10; EGTA, 0.5; MgCl₂, 2; ATP-Mg, 5; GTP-Na, 1; pH 7.25 with KOH (290–300 mOsm, pH: 7.25). Initially, we performed recordings with slices from naïve Venus-VGAT mice to assess the electrophysiological properties of pyramidal neurons vs. interneurons in the lateral OFC. We then recorded spontaneous inhibitory postsynaptic currents (sIPSCs) from SAC and PAE C57BL/6J offspring. Electrophysiological recordings were performed in the whole-cell patch-clamp configuration at a holding membrane potential of –70 mV and a temperature of 32 °C using Axopatch 200B or 700B amplifiers and pClamp10 software (Molecular Devices, Sunnyvale, CA). We used a bath chamber with slice support (Cat # RC-27L; Warner Instruments) to allow flow of ACSF above and below the slice. Spontaneous IPSCs were recorded in presence of 3 mM kynurenic acid using the internal solution described above. The internal solution was allowed to equilibrate with the neuron's cytoplasm for approximately 5 min. Events recorded under these experimental conditions were blocked by 10 µM gabazine, confirming that they were GABA_A receptor mediated. (Tocris, Bristol, U.K.; n =4). The access resistance was between 6 and 33 MΩ and if it changed more than 30%, the recording was rejected. Recordings were analyzed with Mini-Analysis program (Synaptosoft, Decatur, GA).

2.3 Immunohistochemistry.

Behaviorally naïve PAE and SAC mice (n = 8–12/sex/treatment; 1 mouse per litter) were taken at 8–10 weeks of age and perfused using 4% paraformaldehyde (PFA) solution. Brains were extracted and stored in PFA for 48 hours, after which they were washed with 1X phosphate buffered saline (PBS) and sliced on a vibratome (PELCO easiSlicer, Ted Pella, INC. Redding, CA, USA; 50 µm; from ~2.80 mm to ~2.10 mm from bregma). Immunohistochemistry was performed on slices in 24-well plates over a period of 8 days. Slices were blocked and permeabilized for 1.5 hours on a Belly Dancer Platform Shaker (IBI Scientific) in a 300 µL solution of 1% BSA, 5% donkey serum, and 0.2% Triton X-100, brought to volume with PBS. After aspirating the blocking solution, 300 µL of primary antibody solution (monoclonal mouse anti-parvalbumin (dilution: 1:5000 (Cat. No.: PV

235); Swant, Inc., Switzerland; (Schwaller et al., 1999)), polyclonal rabbit anti-somatostatin (dilution: 1:1000 (Cat. No.: T-4103; Peninsula Labs International, Inc., San Carlos, CA, USA; (Ma, Hu, Berrebi, Mathers, & Agmon, 2006)), polyclonal chicken anti-calretinin (dilution: 1:1000; Cat. No.: CH22116; Neuromics, Edina, MN, USA) was added to each well under dim light and slides were covered with aluminum foil and incubated on a shaker in a 4°C walk-in cold room for 7 nights. On the 8th day, the primary antibody was aspirated off and slices were washed 4–5 times with 300 µL PBS for 4 min each. Slices were then blocked and permeabilized for 30 min with 300 µL of a blocking solution without 0.2% Triton X-100. Blocking solution was aspirated out of each well and 300 µL of secondary antibody: Alexa Fluor 488 (donkey anti-mouse IgG; Lot: 1915874; dilution: 1:1000; Invitrogen, Waltham, MA, USA), Alexa Fluor 555 (donkey anti-rabbit IgG; Lot: 1945911; dilution: 1:1000; Invitrogen, Waltham, MA, USA), Alexa Fluor 647 (donkey anti-chicken IgG; Lot: 135091; dilution: 1:1000; Jackson ImmunoResearch, West Grove, PA USA) in blocking solution was added at room temperature for 1.5 hours, after which slices were aspirated and washed 3–4 times with PBS. DAPI (4',6-diamidino-2-phenylindole final concentration: 600 mM; dilution: 1:500) was added and slices were gently agitated for 20 min in dim light and washed with PBS. Slices corresponding to the specific locations of the OFC (Bregma: +2.80, +2.68, +2.46, +2.22) were collected and mounted on a glass microscope slide (4 per slide per subject). Slices were covered with ~150 µL of Fluoromount-G (Thermo Fischer Scientific, USA) and then sealed with glass coverslips, making sure to remove any air bubbles and were stored in a 4°C refrigerator until imaged.

2.4 Unbiased Stereology.

Fluorescence tagged interneuron populations were counted using an Olympus DSU-MBF Stereology System (inverted Olympus IX81) with a mounted Hamamatsu C9100–13 camera and processed using Stereo Investigator (Version 9; MBF Bioscience, VT, USA). Using a DAPI filter to image nuclei, a reference point was placed outside of the orbitofrontal cortex (OFC). Location of OFC was approximated using mouse brain atlas (Paxinos & Franklin, 2001) and a contour was drawn around the OFC (Fig. 1). Using DAPI, the top and bottom of the slices were approximated and a guard zone of 10 µm (5 µm from both coverslip and slide) was added to prevent counting outside of the section. Slices were imaged using filters for various fluorescence (FITC, Texas Red, Cy5, DAPI), using a 40x objective 50% of the contour was analyzed with a grid size of 191.0 µm x 191.0 µm and a counting frame width x height of 135.0 µm x 135.0 µm. Fluorescent interneurons were identified with a digital marker and estimated cell number was calculated by Stereo Investigator using the equation:

$$\sum Q^- * \frac{t}{h} * \frac{1}{asf} * \frac{1}{ssf}$$

Where Q^- is the cells that were marked, t is the mounted thickness of the section, h is the height of the counting frame, asf is the area of the sampling fraction, and ssf is the section of the sampling fraction. For all cell counts, the Gundersen coefficient of error was less than the recommended upper limit value of 0.1 (Gundersen, Jensen, Kieu, & Nielsen, 1999).

2.6 Statistical Analysis.

For slice experiments, statistical analyses were performed with Prism Version 8 (GraphPad Software, San Diego, CA). Data were initially analyzed with the Kolmogorov-Smirnov normality test. The majority of the data passed this normality test and, therefore, were analyzed using unpaired t-tests. Data that did not follow a normal distribution were analyzed with the Mann-Whitney test. A $p < 0.05$ was considered to be statistically significant. Data are presented as mean $\pm$ SEM. The unit of determination is the average of results obtained with slices from a single animal (1–2 cells/animal; 7 mice from 6 litters for SAC and 8 mice from 4 litters for PAE). The investigators were blind to the treatment group assignment of each animal. For interneuron counts, main effects of treatment and sex for all subtypes were compared using two-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test (StatView, SAS Institute, Cary, NC).

3. Results

3.1 Ex vivo electrophysiology.

Recordings with slices from Venus-VGAT mice revealed that lateral OFC pyramidal neurons had a larger membrane capacitance than interneurons (158.9 ± 12 vs 48.78 ± 5.1 pF; $n=6$ neurons from 2 male mice; $p=0.0022$ by Mann-Whitney two-tailed test). However, the membrane resistance was not significantly different between pyramidal neurons (141 ± 25 M Ω) and interneurons (228 ± 62 M Ω ; $n=6$ neurons from 2 male mice; $p=0.2$ by unpaired t-test). Interneurons fired action potentials in response to current injection at a higher frequency than pyramidal neurons (instantaneous firing frequency = 205 ± 18.14 vs 122.5 ± 16.39 Hz in response to 1 nA current injection; $n=6$ neurons from 2 male mice). Therefore, we focused subsequent C57BL/6J mice experiments on neurons with membrane capacitances greater than 100 pF and instantaneous firing frequencies lower than 150 Hz (i.e., which most likely correspond to pyramidal neurons).

Spontaneous IPSCs could be readily recorded in OFC pyramidal neurons from both prenatal alcohol exposed (PAE) and saccharin (SAC) control mice (Fig. 1A). The sIPSC peak amplitude was approximately 50% greater in lateral OFC pyramidal neurons from PAE mice than SAC controls ($p=0.006$ by Mann-Whitney Test; Fig. 1B and C). The sIPSC area was approximately 60% larger in pyramidal neurons from PAE mice ($t(13)=2.769$; $p=0.016$) (Fig. 1B and 1C). The membrane resistance ($t(13)=0.5838$, $p=0.58$), membrane capacitance ($t(13)=0.5863$, $p=0.56$), sIPSC frequency ($t(13)=1.143$, $p=0.27$), and decay time constant (τ_1 ; $t(13)=0.5331$, $p=0.6$) were not significantly different between treatment groups (Fig. 1C). The sIPSC rise times were not significantly different between treatment groups (SAC = 1.130 ± 0.12 ms, $n=7$; PAE = 1.286 ± 0.13 ms, $n=8$; $t(13)=0.8337$, $p=0.41$).

3.2 Interneuron populations in the OFC.

To identify PAE-dependent changes in interneuron populations, we took brain slices from both male and female PAE and SAC mice stained for parvalbumin (PV⁺), somatostatin (SST⁺), calretinin (CR⁺), and DAPI and utilized unbiased stereology software to count cell markers in random, equally spaced locations in the OFC (Fig. 2). A two-way ANOVA revealed a significant main effect of sex for PV⁺ ($F_{1,37}=4.605$; $p=0.0385$; Fig. 3A) and CR⁺

($F_{1,37}=12.247$; $p=0.0012$; Fig. 3C) interneurons, but there was no effect of sex for SST⁺ ($F_{1,37}=4.024$; $p=0.0522$; Fig. 3B) cells. Additionally, we found a significant interaction ($F_{1,37}=4.445$; $p=0.0418$; Fig. 3C) between treatment and sex in cell populations expressing calretinin. Post hoc tests additionally revealed a significant difference of treatment in CR⁺ interneurons ($p<0.05$ by Tukey-Kramer test). Further analysis of stereotaxic regions indicated a significantly greater number of cells in more posterior regions (Bregma: +2.22) of the OFC showed a sex-specific decrease in the PV⁺ ($F_{1,37}=4.568$; $p=0.0392$; Fig. 3D), SST⁺ ($F_{1,37}=12.113$; $p=0.0013$; Fig. 3E) and CR⁺ ($F_{1,37}=8.429$; $p=0.0062$; Fig. 3F) interneuron populations. Similarly, more anterior regions (Bregma: +2.80) of the OFC displayed a main effect of sex ($F_{1,37}=5.850$; $p=0.0206$; Fig. 3F), as well as a treatment x sex interaction ($F_{1,37}=7.652$; $p=0.0088$; Fig. 3F) with regard to CR⁺ cells. Finally, examination of the total number of interneuron markers (PV⁺, SST⁺, & CR⁺) revealed a reduction in the total number of inhibitory interneuron populations in females ($p<0.05$ by Tukey-Kramer test; Fig. 4A) that were specific to different sub-regions of the OFC (Bregma: +2.68, $F_{1,37}=6.103$; $p=0.0182$; Bregma: +2.22, $F_{1,37}=9.229$; $p=0.0044$; Fig. 4B).

4. Discussion

Moderate prenatal exposure to alcohol in mice has been shown to have long-lasting molecular and behavioral effects (Brady et al., 2012; Brady et al., 2013; Marquardt et al., 2020; Marquardt et al., 2014), but the effects that it has on the OFC are relatively unexplored. In the current study, we found that PAE results in significant changes to GABAergic interneuronal populations of the OFC in mice. Using slice electrophysiology, we discovered an increase in the amplitude and area of GABA_A receptor-mediated sIPSCs, specifically in male PAE mice. This is in line with our previous report wherein male PAE mice demonstrated a reduction and delay in low frequency local field potentials (LFPs) associated with unexpected rewards during early stages of reversal learning (Marquardt 2020). Both hippocampal and cortical LFPs have previously been shown to rely on GABAergic input, which when altered, could account for changes in oscillatory activity (Stark 2014, Varga 2014).

In order to understand the effects of PAE on interneuron populations, we utilized immunohistochemical staining and unbiased stereology to quantify specific interneuron populations, which showed no changes in PV⁺, SST⁺, or CR⁺ interneurons in male mice. Analysis of interneuron populations in female mice demonstrated a decrease in numbers of PV⁺ and SST⁺ relative to males, regardless of treatment, but indicated that while CR⁺ numbers were decreased in SAC females, there was a significant increase in this subtype in female PAE mice to a level that approached that of males. Overall, the total population of stained GABAergic interneurons was decreased in female SAC mice, with a slight increase in the average number of cells in PAE females. These data suggest that altered GABAergic tone in the OFC may act in a sex-specific manner, which is surprising as our previous reports indicate that while there is a PAE-specific increase in perseverative behavior during reversal learning, there was no effect of sex as PAE males and females had similar levels of impairment during the early phase of the reversal task. (Marquardt et al., 2014).

Previous research investigating the effects of PAE have utilized various methods, timings, and dosages of exposure (reviewed in Marquardt & Brigman, 2016). The PAE model used here was chosen because it produces BACs (~80 mg/dL) more relevant to ARND, and is targeted to the earlier time points of pregnancy (1st and 2nd trimesters). This drinking-in-the-dark model has been shown previously to produce deficits in behaviors relying on different brain regions (Brady et al., 2012; Marquardt et al., 2014). The hippocampus, in particular, has been shown to be particularly vulnerable to early, moderate exposure to alcohol with both rats and mice demonstrating impairments in both spatial learning and memory, as well as Pavlovian conditioning tasks (Brady et al., 2012; Savage et al., 2010; Staples, Rosenberg, Allen, Porch, & Savage, 2013). However, other studies have found either no (Cullen, Burne, Lavidis, & Moritz, 2014) or meager (D. A. Hamilton et al., 2014) deficits on hippocampal-dependent tasks. Similarly, the moderate PAE paradigm used in this study impairs performance on prefrontal cortical-dependent tasks (Marquardt et al., 2014). As Marquardt and colleagues (2014) have shown, this moderate level of exposure is sufficient to increase maladaptive perseveration, a feature present in human populations affected by FASD (Green et al., 2009). More recently, we have shown that during learning and reversal, PAE mice show significant alterations in pyramidal neuron firing and neuronal recruitment as measured by total number of choice responsive neurons (Marquardt et al., 2020). While these firing alterations were subtle, PAE mice also had reduced oscillatory phase locking to choices during early reversal, indicating that PAE might be altering spike timing in the cortex during flexible behavior. For this reason, we sought to explore the effects of PAE on cortical regions involved in reversal learning, particularly the OFC, in order to better understand the mechanisms behind its effects.

PV⁺ interneurons have been shown to account for ~40% of rodent cortical interneuron subtypes, respectively (reviewed in Rudy et al., 2011). As the most common interneuron subtype, the terms “PV⁺ cell” and “interneuron” are often used interchangeably, particularly in recording studies, as they are strongly correlated with the fast-spiking interneuron pattern (Xu & Callaway, 2009). Previous studies have shown that maternal BACs of ~50 mg/dL during the early postnatal period reduces the number of PV⁺ cells throughout the entire cortex by around 30% (Smiley et al., 2015) while ~20 mg/dL BACs increased PV⁺ interneurons within layer V of the mPFC (Skorput & Yeh, 2016). While we found no change in the number of PV⁺ interneurons in the OFC as a whole, PAE may alter synaptic function of these cells. Since PV⁺ interneurons form perisomatic synapses onto excitatory pyramidal neurons, they effectively modulate action potential firing of these neurons. This may indicate a role for altered PV⁺ function in the altered sIPSCs, as they are likely mediated by action potential-dependent GABA release from PV⁺ interneurons. Our finding that PAE caused an increase in the amplitude and area of sIPSCs in pyramidal neurons of adult offspring suggests that the number and/or density of postsynaptic GABA_A receptors at perisomatic synapses is increased in the lateral OFC. However, the subunit composition of the GABA_A receptors does not appear to be altered as the rise time and decay time constant of the sIPSCs were not significantly affected by PAE. Moreover, sIPSC frequency was not significantly different between treatment groups, indicating that PAE does not alter GABA release driven by spontaneous action potential firing of interneurons. Future studies will be required to determine the mechanism(s) responsible for the increase in sIPSC amplitude and

area, including a detailed analysis of GABA_A receptor subunit expression in OFC pyramidal neurons. Moreover, in light of the results of our IHC experiments, it is also important to systematically investigate whether PAE has sex-dependent effects on the function of different interneuronal subtypes in all OFC antero-posterior sub-regions.

PV⁺ cells have been shown largely to contribute to LFPs in the cortex (Whittington & Traub, 2003) in the high frequency (gamma; 30–80 Hz) range (Sohal, Zhang, Yizhar, & Deisseroth, 2009). However, there is also evidence from studies in the olfactory bulb that SST⁺ and CR⁺ interneurons also contribute to both high and low frequency oscillations (Hardy, Malvaut, Breton-Provencher, & Saghatelian, 2018; Lepousez, Mouret, Loudes, Epelbaum, & Viollet, 2010). As the second most abundant cortical interneuron subtype (~30%), SST⁺ forms axodendritic synapses with primary neurons (Naka & Adesnik, 2016), making it another good inhibitory regulator of excitatory transmission. SST⁺ numbers are reduced in neuropsychiatric disorders, such as a schizophrenia and bipolar disorder (Konradi et al., 2011; Lin & Sibille, 2013; A. Y. Wang et al., 2011), but relatively little is known about how it is affected after PAE with only one study (400–466 mg/dL; gestational day 7) showing dysmorphic SST⁺ interneurons in the ventromedial forebrain (Godin, Dehart, Parnell, O’Leary-Moore, & Sulik, 2011). While our stereological counts were from the entire OFC, *in vitro* recordings were obtained from approx. layer V. However, as previous reports have indicated that interneurons, specifically SST⁺ cells, are more abundant in cortical layer V than in layers II–VI (Naka & Adesnik, 2016; Uematsu et al., 2008; Y. Wang et al., 2004), this suggests any differences in SST⁺ interneuron sub-populations may become more apparent by investigating layer V of the OFC.

CR⁺ cells are nonpyramidal GABAergic neurons distributed in cortical layers I–IV, with the largest proportion located in layer II/III (Reviewed in Druga, 2009). These cells have been shown to constitute ~20% of cortical inhibitory interneurons in rodents (Gonchar, Wang, & Burkhalter, 2007) and ~8% in the human frontal cortex (Gabbott, Jays, & Bacon, 1997). CR⁺ neurons preferentially interact with other GABAergic neurons suggesting that these cells produce an inhibitory effect on other inhibitory neurons (Gulyas, Hajos, & Freund, 1996), therefore, disinhibiting excitatory pyramidal neuron activity, allowing the latter to fire. Interestingly, SST⁺ interneurons have previously been shown to colocalize with CR⁺ cells, particularly in mice (Xu & Callaway, 2009; Xu, Roby, & Callaway, 2006) and we found that ~20% of cells expressing SST⁺ coexpressed CR⁺. This overlap may contribute to our finding that both SST⁺ and CR⁺ are reduced in a sex-dependent manner, as both types were increased in females relative to males. We found that PAE mice, in particular, showed an increase in the number of CR⁺ cells, relative to female SAC mice. This is consistent with previous research looking at alcohol exposure during the third trimester, which showed an increase in CR⁺ interneurons, in the absence of a change in PV⁺ cells (Granato, 2006). We found that these disinhibitory cells are elevated in PAE mice, with a larger effect present in females. This is in line with previous reports that task-specific excitatory signaling is elevated in the OFC following PAE (Marquardt et al., 2020). However, it is important to note that, due to the disinhibitory nature of the cells, CR⁺ interneurons do not contribute to the increased sIPSC amplitudes that we report in pyramidal neurons. This makes comparison of the electrophysiological properties of interneuron → pyramidal neuron and interneuron → interneuron inhibitory transmission after PAE much more important to explore. Since

interneurons are not abundant throughout all cortical layers (Granato, 2006; Granato & Dering, 2018; Hladnik, Dzaja, Darmopil, Jovanov-Milosevic, & Petanjek, 2014; Naka & Adesnik, 2016; Xu et al., 2006), indicating a necessity for future work in this model of mouse PAE to examine relevant cortical layers more closely.

In conclusion, in the current study we found an increase in the duration and area of sIPSCs on pyramidal neurons and a coinciding sex-specific increase in inhibitory interneurons, as well as a treatment-specific effect on cells expressing CR⁺ markers. The data presented in this manuscript will likely serve as an initial step to characterizing the abundance of cellular and molecular alterations that occur in the OFC as a result of moderate PAE model, which may shed more light on its effects.

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References

- Akers KG, Kushner SA, Leslie AT, Clarke L, van der Kooy D, Lerch JP, & Frankland PW (2011). Fetal alcohol exposure leads to abnormal olfactory bulb development and impaired odor discrimination in adult mice. *Mol Brain*, 4, 29. doi:10.1186/1756-6606-4-29 [PubMed: 21736737]
- Audet J-N, & Lefebvre L. (2017). What's flexible in behavioral flexibility? *Behavioral Ecology*, 28(4), 943–947. doi:10.1093/beheco/arx007
- Badanich KA, Mulholland PJ, Beckley JT, Trantham-Davidson H, & Woodward JJ (2013). Ethanol reduces neuronal excitability of lateral orbitofrontal cortex neurons via a glycine receptor dependent mechanism. *Neuropsychopharmacology*, 38(7), 1176–1188. doi:10.1038/npp.2013.12 [PubMed: 23314219]
- Bird CW, Taylor DH, Pinkowski NJ, Chavez GJ, & Valenzuela CF (2018). Long-term Reductions in the Population of GABAergic Interneurons in the Mouse Hippocampus following Developmental Ethanol Exposure. *Neuroscience*, 383, 60–73. doi:10.1016/j.neuroscience.2018.05.003 [PubMed: 29753864]
- Bissonette GB, Bae MH, Suresh T, Jaffe DE, & Powell EM (2010). Astrocyte-mediated hepatocyte growth factor/scatter factor supplementation restores GABAergic interneurons and corrects reversal learning deficits in mice. *J Neurosci*, 30(8), 2918–2923. doi:10.1523/JNEUROSCI.5268-09.2010 [PubMed: 20181589]
- Bissonette GB, Schoenbaum G, Roesch MR, & Powell EM (2015). Interneurons are necessary for coordinated activity during reversal learning in orbitofrontal cortex. *Biol Psychiatry*, 77(5), 454–464. doi:10.1016/j.biopsych.2014.07.023 [PubMed: 25193243]
- Brady ML, Allan AM, & Caldwell KK (2012). A limited access mouse model of prenatal alcohol exposure that produces long-lasting deficits in hippocampal-dependent learning and memory. *Alcohol Clin Exp Res*, 36(3), 457–466. doi:10.1111/j.1530-0277.2011.01644.x [PubMed: 21933200]
- Brady ML, Diaz MR, Iuso A, Everett JC, Valenzuela CF, & Caldwell KK (2013). Moderate prenatal alcohol exposure reduces plasticity and alters NMDA receptor subunit composition in the dentate gyrus. *J Neurosci*, 33(3), 1062–1067. doi:10.1523/JNEUROSCI.1217-12.2013 [PubMed: 23325244]
- Brigman JL, Daut RA, Wright T, Gunduz-Cinar O, Graybeal C, Davis MI, . . . Holmes, A. (2013). GluN2B in corticostriatal circuits governs choice learning and choice shifting. *Nat Neurosci*, 16(8), 1101–1110. doi:10.1038/nn.3457 [PubMed: 23831965]

- Burkhalter A. (2008). Many specialists for suppressing cortical excitation. *Front Neurosci*, 2(2), 155–167. doi:10.3389/neuro.01.026.2008 [PubMed: 19225588]
- Casale AE, Foust AJ, Bal T, & McCormick DA (2015). Cortical Interneuron Subtypes Vary in Their Axonal Action Potential Properties. *J Neurosci*, 35(47), 15555–15567. doi:10.1523/JNEUROSCI.1467-13.2015 [PubMed: 26609152]
- Coles CD, Kable JA, Keen CL, Jones KL, Wiertelicki W, Granovska IV, . . . Cifasd. (2015). Dose and Timing of Prenatal Alcohol Exposure and Maternal Nutritional Supplements: Developmental Effects on 6-Month-Old Infants. *Matern Child Health J*, 19(12), 2605–2614. doi:10.1007/s10995-015-1779-x [PubMed: 26164422]
- Cullen CL, Burne TH, Lavidis NA, & Moritz KM (2014). Low dose prenatal alcohol exposure does not impair spatial learning and memory in two tests in adult and aged rats. *PLoS One*, 9(6), e101482. doi:10.1371/journal.pone.0101482 [PubMed: 24978807]
- Cuzon VC, Yeh PW, Yanagawa Y, Obata K, & Yeh HH (2008). Ethanol consumption during early pregnancy alters the disposition of tangentially migrating GABAergic interneurons in the fetal cortex. *J Neurosci*, 28(8), 1854–1864. doi:10.1523/JNEUROSCI.5110-07.2008 [PubMed: 18287502]
- DeFelipe J, Lopez-Cruz PL, Benavides-Piccione R, Bielza C, Larranaga P, Anderson S, . . . Ascoli GA (2013). New insights into the classification and nomenclature of cortical GABAergic interneurons. *Nat Rev Neurosci*, 14(3), 202–216. doi:10.1038/nrn3444 [PubMed: 23385869]
- Diamond A. (2013). Executive functions. *Annu Rev Psychol*, 64, 135–168. doi:10.1146/annurev-psych-113011-143750 [PubMed: 23020641]
- Druga R. (2009). Neurocortical inhibitory system. *Folia Biologica*, 55, 201–217. [PubMed: 20163769]
- Ethen MK, Ramadhani TA, Scheuerle AE, Canfield MA, Wyszynski DF, Druschel CM, . . . National Birth Defects Prevention, S. (2009). Alcohol consumption by women before and during pregnancy. *Matern Child Health J*, 13(2), 274–285. doi:10.1007/s10995-008-0328-2 [PubMed: 18317893]
- Ferguson BR, & Gao WJ (2018). PV Interneurons: Critical Regulators of E/I Balance for Prefrontal Cortex-Dependent Behavior and Psychiatric Disorders. *Front Neural Circuits*, 12, 37. doi:10.3389/fncir.2018.00037 [PubMed: 29867371]
- Floresco SB, Zhang Y, & Enomoto T. (2009). Neural circuits subserving behavioral flexibility and their relevance to schizophrenia. *Behav Brain Res*, 204(2), 396–409. doi:10.1016/j.bbr.2008.12.001 [PubMed: 19110006]
- Gabbott PLA, Jays PRL, & Bacon SJ (1997). Calretinin neurons in human medial prefrontal cortex (Areas 24a,b,c, 32', and 25). *J Comparative Neurology*, 381, 389–410.
- Georgieff MK, Tran PV, & Carlson ES (2018). Atypical fetal development: Fetal alcohol syndrome, nutritional deprivation, teratogens, and risk for neurodevelopmental disorders and psychopathology. *Dev Psychopathol*, 30(3), 1063–1086. doi:10.1017/S0954579418000500 [PubMed: 30068419]
- Godin EA, Dehart DB, Parnell SE, O'Leary-Moore SK, & Sulik KK (2011). Ventromedian forebrain dysgenesis follows early prenatal ethanol exposure in mice. *Neurotoxicol Teratol*, 33(2), 231–239. doi:10.1016/j.ntt.2010.11.001 [PubMed: 21074610]
- Gonchar Y, Wang Q, & Burkhalter A. (2007). Multiple distinct subtypes of GABAergic neurons in mouse visual cortex identified by triple immunostaining. *Front Neuroanat*, 1, 3. doi:10.3389/neuro.05.003.2007 [PubMed: 18958197]
- Granato A. (2006). Altered organization of cortical interneurons in rats exposed to ethanol during neonatal life. *Brain Res*, 1069(1), 23–30. doi:10.1016/j.brainres.2005.11.024 [PubMed: 16386714]
- Granato A, & Dering B. (2018). Alcohol and the Developing Brain: Why Neurons Die and How Survivors Change. *Int J Mol Sci*, 19(10). doi:10.3390/ijms19102992
- Green CR, Mihic AM, Nikkel SM, Stade BC, Rasmussen C, Munoz DP, & Reynolds JN (2009). Executive function deficits in children with fetal alcohol spectrum disorders (FASD) measured using the Cambridge Neuropsychological Tests Automated Battery (CANTAB). *J Child Psychol Psychiatry*, 50(6), 688–697. doi:10.1111/j.1469-7610.2008.01990.x [PubMed: 19175817]
- Gruner P, & Pittenger C. (2017). Cognitive inflexibility in Obsessive-Compulsive Disorder. *Neuroscience*, 345, 243–255. doi:10.1016/j.neuroscience.2016.07.030 [PubMed: 27491478]

- Gulyas AI, Hajos N, & Freund TF (1996). Interneurons containing calretinin are specialized to control other interneurons in the rat hippocampus. *J Neurosci*, 16(10), 3397–3411.
- Gundersen HJG, Jensen EBV, Kieu K, & Nielsen J. (1999). The efficiency of systematic sampling in stereology - reconsidered. *Journal of Microscopy*, 193(3), 199–211. [PubMed: 10348656]
- Hamilton DA, & Brigman JL (2015). Behavioral flexibility in rats and mice: contributions of distinct frontocortical regions. *Genes Brain Behav*, 14(1), 4–21. doi:10.1111/gbb.12191 [PubMed: 25561028]
- Hamilton DA, Magcalas CM, Barto D, Bird CW, Rodriguez CI, Fink BC, . . . Savage DD (2014). Moderate prenatal alcohol exposure and quantification of social behavior in adult rats. *J Vis Exp*(94). doi:10.3791/52407
- Hamilton GF, Hernandez IJ, Krebs CP, Bucko PJ, & Rhodes JS (2017). Neonatal alcohol exposure reduces number of parvalbumin-positive interneurons in the medial prefrontal cortex and impairs passive avoidance acquisition in mice deficits not rescued from exercise. *Neuroscience*, 352, 52–63. doi:10.1016/j.neuroscience.2017.03.058 [PubMed: 28391014]
- Hardy D, Malvaut S, Breton-Provencher V, & Saghatelian A. (2018). The role of calretinin-expressing granule cells in olfactory bulb functions and odor behavior. *Sci Rep*, 8(1), 9385. doi:10.1038/s41598-018-27692-8 [PubMed: 29925844]
- Hladnik A, Dzaja D, Darmopil S, Jovanov-Milosevic N, & Petanjek Z. (2014). Spatio-temporal extension in site of origin for cortical calretinin neurons in primates. *Front Neuroanat*, 8, 50. doi:10.3389/fnana.2014.00050 [PubMed: 25018702]
- Izquierdo A, Brigman JL, Radke AK, Rudebeck PH, & Holmes A. (2017). The neural basis of reversal learning: An updated perspective. *Neuroscience*, 345, 12–26. doi:10.1016/j.neuroscience.2016.03.021 [PubMed: 26979052]
- Izquierdo A, & Jentsch JD (2012). Reversal learning as a measure of impulsive and compulsive behavior in addictions. *Psychopharmacology*, 219(2), 607–620. doi:10.1007/s00213-011-2579-7 [PubMed: 22134477]
- Jarmasz JS, Basalah DA, Chudley AE, & Del Bigio MR (2017). Human Brain Abnormalities Associated With Prenatal Alcohol Exposure and Fetal Alcohol Spectrum Disorder. *J Neuropathol Exp Neurol*, 76(9), 813–833. doi:10.1093/jnen/nlx064 [PubMed: 28859338]
- Kingdon D, Cardoso C, & McGrath JJ (2016). Research Review: Executive function deficits in fetal alcohol spectrum disorders and attention-deficit/hyperactivity disorder - a meta-analysis. *J Child Psychol Psychiatry*, 57(2), 116–131. doi:10.1111/jcpp.12451 [PubMed: 26251262]
- Konradi C, Yang CK, Zimmerman EI, Lohmann KM, Gresch P, Pantazopoulos H, . . . Heckers S. (2011). Hippocampal interneurons are abnormal in schizophrenia. *Schizophr Res*, 131(1–3), 165–173. doi:10.1016/j.schres.2011.06.007 [PubMed: 21745723]
- Lepousez G, Mouret A, Loudes C, Epelbaum J, & Viollet C. (2010). Somatostatin contributes to in vivo gamma oscillation modulation and odor discrimination in the olfactory bulb. *J Neurosci*, 30(3), 870–875. doi:10.1523/JNEUROSCI.4958-09.2010 [PubMed: 20089895]
- Lin LC, & Sibille E. (2013). Reduced brain somatostatin in mood disorders: a common pathophysiological substrate and drug target? *Front Pharmacol*, 4, 110. doi:10.3389/fphar.2013.00110 [PubMed: 24058344]
- Ma Y, Hu H, Berrebi AS, Mathers PH, & Agmon A. (2006). Distinct subtypes of somatostatin-containing neocortical interneurons revealed in transgenic mice. *J Neurosci*, 26(19), 5069–5082. doi:10.1523/JNEUROSCI.0661-06.2006 [PubMed: 16687498]
- Madden JT, Thompson SM, Magcalas CM, Wagner JL, Hamilton DA, Savage DD, . . . Pentkowski NS (2020). Moderate prenatal alcohol exposure reduces parvalbumin expressing GABAergic interneurons in the dorsal hippocampus of adult male and female rat offspring. *Neurosci Lett*, 718, 134700. doi:10.1016/j.neulet.2019.134700 [PubMed: 31874217]
- Marquardt K, & Brigman JL (2016). The impact of prenatal alcohol exposure on social, cognitive and affective behavioral domains: Insights from rodent models. *Alcohol*, 51, 1–15. doi:10.1016/j.alcohol.2015.12.002 [PubMed: 26992695]
- Marquardt K, Cavanagh JF, & Brigman JL (2020). Alcohol exposure in utero disrupts corticostriatal coordination required for behavioral flexibility. *Neuropharmacology*, 162, 107832. doi:10.1016/j.neuropharm.2019.107832 [PubMed: 31678398]

- Marquardt K, Sigdel R, Caldwell K, & Brigman JL (2014). Prenatal ethanol exposure impairs executive function in mice into adulthood. *Alcohol Clin Exp Res*, 38(12), 2962–2968. doi:10.1111/acer.12577 [PubMed: 25581651]
- Naka A, & Adesnik H. (2016). Inhibitory Circuits in Cortical Layer 5. *Front Neural Circuits*, 10, 35. doi:10.3389/fncir.2016.00035 [PubMed: 27199675]
- Negron-Oyarzo I, Aboitiz F, & Fuentealba P. (2016). Impaired Functional Connectivity in the Prefrontal Cortex: A Mechanism for Chronic Stress-Induced Neuropsychiatric Disorders. *Neural Plast*, 2016, 7539065. doi:10.1155/2016/7539065 [PubMed: 26904302]
- Nirgudkar P, Taylor DH, Yanagawa Y, & Valenzuela CF (2016). Ethanol exposure during development reduces GABAergic/glycinergic neuron numbers and lobule volumes in the mouse cerebellar vermis. *Neurosci Lett*, 632, 86–91. doi:10.1016/j.neulet.2016.08.039 [PubMed: 27565053]
- Patten AR, Fontaine CJ, & Christie BR (2014). A comparison of the different animal models of fetal alcohol spectrum disorders and their use in studying complex behaviors. *Front Pediatr*, 2, 93. doi:10.3389/fped.2014.00093 [PubMed: 25232537]
- Paxinos G, & Franklin KBJ (2001). *The mouse brain in stereotaxic coordinates*. London: Academic Press.
- Popova S, Lange S, Probst C, Gmel G, & Rehm J. (2017). Estimation of national, regional, and global prevalence of alcohol use during pregnancy and fetal alcohol syndrome: a systematic review and meta-analysis. *The Lancet Global Health*, 5(3), e290–e299. doi:10.1016/s2214-109x(17)30021-9 [PubMed: 28089487]
- Remijnse PL, van den Heuvel OA, Nielen MM, Vriend C, Hendriks GJ, Hoogendijk WJ, . . . Veltman DJ (2013). Cognitive inflexibility in obsessive-compulsive disorder and major depression is associated with distinct neural correlates. *PLoS One*, 8(4), e59600. doi:10.1371/journal.pone.0059600 [PubMed: 23637737]
- Riley EP, Infante MA, & Warren KR (2011). Fetal alcohol spectrum disorders: an overview. *Neuropsychol Rev*, 21(2), 73–80. doi:10.1007/s11065-011-9166-x [PubMed: 21499711]
- Rudy B, Fishell G, Lee S, & Hjerling-Leffler J. (2011). Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons. *Dev Neurobiol*, 71(1), 45–61. doi:10.1002/dneu.20853 [PubMed: 21154909]
- Saito M, Smiley JF, Hui M, Masiello K, Betz J, Ilina M, . . . Wilson DA (2019). Neonatal Ethanol Disturbs the Normal Maturation of Parvalbumin Interneurons Surrounded by Subsets of Perineuronal Nets in the Cerebral Cortex: Partial Reversal by Lithium. *Cereb Cortex*, 29(4), 1383–1397. doi:10.1093/cercor/bhy034 [PubMed: 29462278]
- Savage DD, Rosenberg MJ, Wolff CR, Akers KG, El-Emawy A, Staples MC, . . . Hamilton DA (2010). Effects of a novel cognition-enhancing agent on fetal ethanol-induced learning deficits. *Alcohol Clin Exp Res*, 34(10), 1793–1802. doi:10.1111/j.1530-0277.2010.01266.x [PubMed: 20626729]
- Schwaller B, Dick J, Dhoot G, Carroll S, Vrbova G, P N, . . . Celio M. (1999). Prolonged contraction-relaxation cycle of fast-twitch muscles in parvalbumin knockout mice. *Am J Physiol*, 276(2), C395–403. [PubMed: 9950767]
- Shenoda BB (2017). An Overview of the Mechanisms of Abnormal GABAergic Interneuronal Cortical Migration Associated with Prenatal Ethanol Exposure. *Neurochem Res*, 42(5), 1279–1287. doi:10.1007/s11064-016-2169-5 [PubMed: 28160199]
- Singer AB, Alyslworth AS, Cordero C, Croen LA, DiGiuseppi C, Fallin MD, . . . Daniels JL (2017). Prenatal alcohol exposure in relation to autism spectrum disorder: findings from the study to explore early development (SEED). *Paediatric and Perinatal Epidemiology*, 31, 573–582. [PubMed: 28881390]
- Skorput AG, & Yeh HH (2016). Chronic Gestational Exposure to Ethanol Leads to Enduring Aberrances in Cortical Form and Function in the Medial Prefrontal Cortex. *Alcohol Clin Exp Res*, 40(7), 1479–1488. doi:10.1111/acer.13107 [PubMed: 27242215]
- Smiley JF, Saito M, Bleiwas C, Masiello K, Ardekani B, Guilfoyle DN, . . . Vadasz C. (2015). Selective reduction of cerebral cortex GABA neurons in a late gestation model of fetal alcohol spectrum disorder. *Alcohol*, 49(6), 571–580. doi:10.1016/j.alcohol.2015.04.008 [PubMed: 26252988]

- Sohal VS, Zhang F, Yizhar O, & Deisseroth K. (2009). Parvalbumin neurons and gamma rhythms enhance cortical circuit performance. *Nature*, 459(7247), 698–702. doi:10.1038/nature07991 [PubMed: 19396159]
- Stalnaker TA, Cooch NK, & Schoenbaum G. (2015). What the orbitofrontal cortex does not do. *Nat Neurosci*, 18(5), 620–627. doi:10.1038/nn.3982 [PubMed: 25919962]
- Staples MC, Rosenberg MJ, Allen NA, Porch MW, & Savage DD (2013). Impact of combined prenatal ethanol and prenatal stress exposure on anxiety and hippocampal-sensitive learning in adult offspring. *Alcohol Clin Exp Res*, 37(12), 2039–2047. doi:10.1111/acer.12190 [PubMed: 23915215]
- Stark E, Eichler R, Roux L, Fujisawa S, Rotstein HG, & Buzsaki G, (2013). Inhibition-Induced Theta Resonance in Cortical Circuits. *Neuron*, 80(4), 1263–1276. doi:10106/j.neuron.2013.09.033 [PubMed: 24314731]
- Tremblay R, Lee S, & Rudy B. (2016). GABAergic Interneurons in the Neocortex: From Cellular Properties to Circuits. *Neuron*, 91(2), 260–292. doi:10.1016/j.neuron.2016.06.033 [PubMed: 27477017]
- Uematsu M, Hirai Y, Karube F, Ebihara S, Kato M, Abe K, . . . Kawaguchi Y. (2008). Quantitative chemical composition of cortical GABAergic neurons revealed in transgenic venus-expressing rats. *Cereb Cortex*, 18(2), 315–330. doi:10.1093/cercor/bhm056 [PubMed: 17517679]
- Varga C, Oijala M, Lish J, Szabo GG, Bezair M, Marchionni I, Golshani P, Soltesz I. (2014) Functional fission of parvalbumin interneuron classes during fast network events. *eLife* 2014;3:e04006, doi: 10.7554/eLife.04006
- Wang AY, Lohmann KM, Yang CK, Zimmerman EI, Pantazopoulos H, Herring N, . . . Konradi C. (2011). Bipolar disorder type 1 and schizophrenia are accompanied by decreased density of parvalbumin- and somatostatin-positive interneurons in the parahippocampal region. *Acta Neuropathol*, 122(5), 615–626. doi:10.1007/s00401-011-0881-4 [PubMed: 21968533]
- Wang Y, Kakizaki T, Sakagami H, Saito K, Ebihara S, Kato M, . . . Yanagawa Y. (2009). Fluorescent labeling of both GABAergic and glycinergic neurons in vesicular GABA transporter (VGAT)-venus transgenic mouse. *Neuroscience*, 164(3), 1031–1043. doi:10.1016/j.neuroscience.2009.09.010 [PubMed: 19766173]
- Wang Y, Toledo-Rodriguez M, Gupta A, Wu C, Silberberg G, Luo J, & Markram H. (2004). Anatomical, physiological and molecular properties of Martinotti cells in the somatosensory cortex of the juvenile rat. *J Physiol*, 561(Pt 1), 65–90. doi:10.1113/jphysiol.2004.073353 [PubMed: 15331670]
- Whittington MA, & Traub RD (2003). Interneuron diversity series: inhibitory interneurons and network oscillations in vitro. *Trends Neurosci*, 26(12), 676–682. doi:10.1016/j.tins.2003.09.016 [PubMed: 14624852]
- Xu X, & Callaway EM (2009). Laminar specificity of functional input to distinct types of inhibitory cortical neurons. *J Neurosci*, 29(1), 70–85. doi:10.1523/JNEUROSCI.4104-08.2009 [PubMed: 19129386]
- Xu X, Roby KD, & Callaway EM (2006). Mouse cortical inhibitory neuron type that coexpresses somatostatin and calretinin. *J Comp Neurol*, 499(1), 144–160. doi:10.1002/cne.21101 [PubMed: 16958092]
- Young C, & Olney JW (2006). Neuroapoptosis in the infant mouse brain triggered by a transient small increase in blood alcohol concentration. *Neurobiol Dis*, 22(3), 548–554. doi:10.1016/j.nbd.2005.12.015 [PubMed: 16459096]

Highlights:

- Moderate PAE alters GABAergic tone in the OFC.
- Female SAC mice have fewer inhibitory interneurons in the OFC.
- Axo-somatic, parvalbumin-expressing and axo-dendritic, somatostatin-expressing interneurons are similar in population size in the OFC between both PAE and SAC control mice.
- Disinhibitory, calretinin-expressing interneurons are increased following PAE, particularly in females.
- Highlights the effects of PAE on cortical GABAergic interneurons in cortical areas involved in aspects of executive control.

Fig 1. Effect of PAE on GABA_A receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) in OFC pyramidal neurons.

A. Exemplar traces of sIPSC slice recordings from control (saccharin, SAC) and prenatal alcohol-exposed (PAE) mice. B. Superimposed average traces from the same recordings illustrated in A showing the PAE-induced increase in sIPSC amplitude (black trace= SAC; red trace=PAE; note the expanded time scale). C. Summary of the effect of PAE on membrane resistance, membrane capacitance, sIPSC amplitude, sIPSC frequency, sIPSC decay time constant (τ_1), and sIPSC area. ** $p=0.006$ by Mann-Whitney Test; * $p=0.016$ by two-tailed unpaired t-test.

Fig 2. Unbiased stereology for interneuron counting in the OFC.

A. OFC contours are freely drawn in Stereo Investigator based on images in Paxinos and Franklin (2001). AP: +2.22 is the most caudal slices used. *B.* Labeled cells within each counting frame or touching the green border are manually marked by hand, while cells outside of the counting frame or touching the red border are unmarked, and therefore uncounted. O = PV + cells, + = SST+ cells, x = CR+ cells

Fig 3. Comparison of inhibitory interneuron populations in tissue triple stained for PV⁺, SST⁺, and CR⁺ cells between male and female PAE and SAC control mice.

A, D. Female mice, across both PAE and SAC treatments, have significantly fewer PV⁺ cells in the OFC, particularly in the posterior OFC. *B, E.* The total number of SST⁺ cells in the OFC is non-significantly reduced in female PAE and SAC mice in the posterior OFC. *C, F.* CR⁺ interneurons are reduced in female SAC mice, but are increased in PAE females.

* $p < 0.05$ by two-way ANOVA; # $p < 0.05$ by Tukey-Kramer post hoc test.

Fig 4. Average number of all cells expressing inhibitory interneuron markers (PV⁺/SST⁺/CR⁺) in mouse OFC.

A. Across treatments, females have fewer cells expressing inhibitory interneuron markers. *B.* Female mice across treatments display fewer inhibitory interneurons at specific stereotaxic regions. * $p < 0.05$ by two-way ANOVA.