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Nucleus reuniens of midline thalamus of a rat is specifically damaged after early postnatal alcohol exposure.

Z.H. Gursky¹, L.M. Savage², and A.Y. Klintsova^{1,*}

¹Department of Psychological & Brain Sciences, University of Delaware, Newark, DE 19716, USA

²Department of Psychology, Binghamton University (State University of New York), Binghamton, NY 13902, USA

Abstract

Individuals diagnosed with fetal alcohol spectrum disorders (FASD) often display behavioral impairments in executive functioning. Mechanistic studies have implicated coordination between prefrontal cortex and hippocampus (through thalamic nucleus reuniens) as essential for such executive functions. The current study is the first to report the long-term neuroanatomical alterations to ventral midline thalamus after alcohol exposure on postnatal days 4-9 (a rodent model of binge drinking during third trimester of human pregnancy). Alcohol added to milk formula was administered to female Long Evans rat pups on postnatal days 4-9 (5.25 g/kg/day of ethanol, intragastric intubation). Control animals were intubated without administration of liquid. In adulthood, brains were immunohistochemically labeled for a neuronal marker (NeuN) conjugated with Cy3 fluorophore and stained with Hoechst33342 to visualize nuclei. Total non-neuronal cell number (NeuN⁻/Hoechst⁺) and neuron number (NeuN⁺/Hoechst⁺), and total volume were estimated using unbiased stereology in two neighboring midline thalamic nuclei: reuniens and rhomboid. Estimates were analyzed using linear mixed modeling to account for animal and litter as clustering variables. A 21% reduction in total neuron number (resulting in altered neuron-to-non-neuron ratio) and 18% reduction in total volume, were found exclusively in thalamic nucleus reuniens in rats exposed to ethanol. Non-neuronal cell number was not changed in reuniens. No ethanol-induced changes on any measures were observed in rhomboid nucleus. These specific neuroanatomical alterations provide a necessary foundation for further examination of circuit-level alterations that occur in FASD.

Keywords

FASD; Thalamus; Stereology; Nucleus reuniens; Rhomboid nucleus; Immunofluorescence; Immunohistochemistry; Linear mixed modeling

*Corresponding Author (needs the full name/address): Anna Y. Klintsova, Department of Psychological & Brain Sciences, University of Delaware, Newark, DE 19716, USA. klintsov@udel.edu (AYK).

Statement of conflicts of interest

The authors have no conflicts of interest to declare.

Introduction

Alcohol consumption is prevalent among women of childbearing age in the United States [1], with many of them continuing to consume alcohol between conception, recognition of pregnancy and advanced pregnancy [2]. Prenatal alcohol exposure often results in a fetal alcohol spectrum disorder (FASD), the prevalence of which is estimated between 1-10% for most countries [3]. FASD-afflicted individuals display impairments in a subset of cognitive tasks collectively referred to as “executive function,” which include, but are not limited to, cognitive flexibility, deductive reasoning, problem solving, and concept formation [4].

Early postnatal alcohol exposure in rats has consistently produced damage to brain structures critical for learning, memory, and cognition, such as the hippocampus (HPC) [5] and medial prefrontal cortex (mPFC) [6]. These two structures form a functional circuit along with the thalamus and have the strongest reciprocal connectivity through nucleus reuniens (Re) of the ventral midline thalamus. Despite its role in regulating activity of the mPFC-HPC circuit, Re as a critical nucleus within ventral midline thalamus has not yet been examined in models of developmental alcohol exposure. No published data exists, to date, characterizing nucleus-specific damage to the ventral midline thalamus in either human studies or rodent models of FASD. Therefore, the current study was undertaken to test the hypothesis that Re structural integrity is affected by third trimester alcohol exposure, implicating dysfunction in the entire mPFC-Re-HPC circuit in FASD. We examined the long-term impact of early postnatal alcohol exposure on Re of midline thalamus, a critical substrate for mPFC-HPC interactions, in female rats. We compared Re with the neighboring thalamic rhomboid nucleus (Rh) to determine the specificity of alcohol’s influence on ventral midline thalamic development.

Methods

Experimental Subjects

All animal procedures were performed in accordance with the University of Delaware animal care committee’s regulations. A total of 13 female Long Evans rats from 4 litters were used in the current study. To account for uneven representation of litters in data, all statistical analyses account for litter as a clustering variable, as suggested by Galbraith, Daniel [7]. All animals were obtained from in-house breeding colony at the University of Delaware. On postnatal day (PD) 3, pups within a litter were randomly placed into one of two experimental groups: alcohol exposed (AE) or sham-intubated (SI). All animals were weighed daily at 9AM. Alcohol exposure was performed daily from PD4-9 via intragastric intubation over 2 doses, 2 hours apart (9AM and 11AM). Intubation was performed by applying a small amount of corn oil to the tip of PE10 surgical tubing, which would gently be placed down the esophagus of the pup. SI animals were intubated for 10-15 seconds (the approximate amount of time required to intubate and administer milk substitute or alcohol in milk substitute to an AE pup) but did not have milk substitute or alcohol administered. 5.25 g/kg/day of 11.9% v/v ethanol in a milk substitute was administered to AE animals, in addition to 1 daily supplemental dose of milk substitute (2 supplemental doses on PD 4) at 2 hours after the second ethanol administration (i.e., 1PM on PD4-9 and 3PM on PD4 only) to prevent confounding weight differences. To further minimize weight differences (i.e., weight

reduction in AE animals) in a manner consistent with a large number of previous publications using this model [8–10], SI control animals were not administered any liquid (e.g., a calorically similar solution or milk substitute). AE and SI animals would be removed from the mother for no more than 20 minutes per session of intubation. Blood samples were collected on PD 4 at exactly 90 minutes following the second ethanol administration (approximately 12:30PM) [11]. BAC in centrifuged plasma of AE pups was analyzed using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA). SI animals also had blood drawn, to account for the stressful effect of this procedure, but their blood samples were not analyzed. All animals were earpunched on PD 9 for identification purposes. On PD 23, all animals were weaned and subsequently housed in social cages of 2-3 same-sex animals.

Tissue Preparation, Histology, and Immunohistochemistry

On PD 72, female rats were anesthetized with gas isoflurane followed by ketamine-xylazine administration via intraperitoneal injection. Rats were perfused with heparinized 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1M PBS, (pH = 7.2). Brains were postfixed in 4% paraformaldehyde in 0.1M PBS for 24 hrs, then equilibrated with 30% sucrose in fixative solution. Brains were sectioned coronally at 40µm using a cryostat (CM3050S; Leica Biosystems, Wetzlar, Germany) and serial sections were collected in a cryoprotectant buffer solution.

Every 6th serial section containing Re and Rh was used for free-floating immunohistochemical processing. Immunofluorescent staining against the NeuN protein and counterstaining with Hoechst33342 were used to identify neuronal (NeuN⁺/Hoechst⁺) and non-neuronal cells (NeuN⁻/Hoechst⁺) in Re and Rh. Tissue was first rinsed in 0.1M tris-buffer solution (TBS; pH = 7.40) and blocked in a solution of 3 % donkey serum (S30; Merck Millipore, Burlington, MA) and 0.5% Triton X-100 (BP-151; Fisher Scientific, Hampton, NH) in 0.1M TBS for 1 hr. Then, tissue was incubated in primary monoclonal anti-NeuN antibody raised in mouse (Millipore Cat# MAB377, RRID:AB_2298772) in TBS/donkey serum/Triton-X blocking solution at 1:500 dilution for 48 hrs at 4°C. Next, tissue was washed in 0.1M TBS then incubated in a Cy3-conjugated anti-mouse polyclonal secondary antibody raised in donkey (Jackson ImmunoResearch Labs Cat# 715-165-151, RRID:AB_2315777) in blocking solution at 1:250 dilution for 2 hrs. Tissue was washed once more in 0.1M TBS prior to slide mounting in dH₂O. After air drying for 1 hr, tissue was rehydrated with 0.1M PBS and stained with Hoechst33342 (H3570; ThermoFisher Scientific, Waltham, MA) in 0.1M PBS (1:1000) for 5 min. Tissue then washed with 0.1M PBS prior to cover-slipping with Vectashield Anti-fade Mounting Medium (H-1000; Vector Laboratories, Burlingame, CA). After allowing mounting medium to set for 12 hrs, slides were sealed with clear coat nail polish (Insta-Dri 106; Sally Hansen) and stored at 4°C.

Unbiased Stereological Quantification

Cell number and volume within each thalamic nucleus was quantified using the optical fractionator [12] workflow in Stereo Investigator software ([Stereo Investigator](#), RRID:SCR_002526). Sections, approximately every 240µm, containing Re and Rh were exhaustively mounted (8-12 sections per animal). Tissue was imaged using an

Axioskop2Plus outfitted with a 5× Plan-Neofluar objective (Carl Zeiss Inc., Thornwood, NY) for contouring the regions of interest and a 63× oil-immersion Plan-Neofluar (Carl Zeiss Inc., Thornwood, NY) for immunofluorescent quantification. The regions of interest were outlined according to contours and histological sections of Paxinos and Watson [13], and demonstrated in fig. 1A. The following stereological parameters were utilized in the optical fractionator probe: 30µm × 30µm counting frame, 210µm × 210µm grid size, 25.00µm disector height with 1.00µm guard zones, and 1/6 section counting fraction. Mean coefficients of error (Gundersen, m=1) were below recommended 0.1.

Statistical Analyses

All statistical analyses were performed using RStudio (RStudio, RRID:SCR_000432) with R version 3.4.3 [14] running the “tidyverse” [15] and “lmerTest” [16] packages. All data were analyzed using the “lmer” function from the “lmerTest” package to allow for linear mixed model (LMM) analysis, which appropriately accounts for clustering of outcome variables (i.e., multiple observations per litter, or repeated measures within-animal) in data sets with “smaller” sample sizes or non-normal distributions [7]. Regression coefficient estimates (β) are expressed as the estimate $\pm$ standard error. $\alpha = 0.050$ was used for all statistical analyses.

Weight data were stratified into weight during dosing (PD4-9) and adult weight (PD72). Weight during dosing was analyzed using postnatal treatment and days since the start of dosing as fixed effect predictors, and animal and litter as random effects (i.e., clustering variable). Weight on PD72 was analyzed using postnatal treatment as a fixed effect predictor, and litter as a random effect.

Each neuroanatomical variable (estimated total neuron number, total non-neuron cell number, neuronal ratio, and total volume) was analyzed using a linear mixed model with structure (Re, Rh) and postnatal treatment (SI, AE) as fixed effects predictors and litter and animal as random effects.

Results

AE animals achieved a peak BAC of 378.60 mg/dL $\pm$ 37.64 mg/dL (mean $\pm$ SEM) on PD4, 90 minutes following the final alcohol administration that day (table 1). These levels are similar to many previous studies using this method and timing of alcohol exposure, which typically range between 320-420 mg/dL [8, 9, 17].

All animals gained weight every day during the dosing period (i.e., PD4-9) ($\beta = 1.78 \pm 0.05$, $t(63) = 34.32$, $p < 0.001$). While AE animals did not differ in weight from SI animals at the start of dosing ($\beta = 0.24 \pm 0.65$, $t(13.63) = 0.36$, $p = 0.722$), there was a significant treatment-by-age interaction ($\beta = -0.53 \pm 0.08$, $t(63) = -6.40$, $p < 0.001$) indicating that AE animals did not gain as much weight daily as SI animals. There was no significant effect of alcohol exposure on weight at PD72 ($\beta = 19.00 \pm 9.17$, $t(8.15) = 2.07$, $p = 0.071$). Average weights can be found in table 1.

Analysis of neuron number in ventral midline thalamus (fig. 1B) revealed an interaction between postnatal treatment and structure: AE animals had less neurons in Re than SI

animals ($\beta = -10306 \pm 4636$, $t(11) = -2.22$, $p = 0.048$). In contrast, there was no effect of postnatal treatment on the number of neurons in Rh ($\beta = -3584 \pm 3988$, $t(16.83) = -0.90$, $p = 0.382$). In both control and alcohol-exposed brains Re had more neurons than Rh ($\beta = 17225 \pm 2875$, $t(11) = 5.99$, $p < 0.001$), although this difference was less pronounced in AE animals (see interaction indicating AE-induced loss of approximately 10306 neurons, above), indicating selective neuron loss in Re, but not Rh.

In contrast to neuron number, there was no significant main effect of AE ($\beta = -11474 \pm 6859$, $t(14.53) = -1.67$, $p = 0.116$) nor interaction between postnatal treatment and structure ($\beta = -4908 \pm 6504$, $t(11) = -0.76$, $p = 0.466$) on non-neuronal cell number (fig. 1C). The Re of SI animals had more non-neuronal cells than the Rh ($\beta = 38725 \pm 4033$, $t(11) = 9.60$, $p < 0.001$), a pattern which was similar in AE animals. This indicates that there was no significant difference in non-neuronal cell number in either Rh or Re between SI and AE animals.

As a result of neuronal loss in Re, but not Rh due to alcohol exposure, and no differences in non-neuronal cell number, we examined the neuronal ratio (i.e., percentage of cells in a given structure that were neuronal) (fig. 1D). There was an interaction between postnatal treatment and structure revealing that Re of AE animals had a reduced neuronal ratio relative to Re of SI animals ($\beta = -7.26 \pm 2.67$, $t(11) = -2.72$, $p = 0.020$). In contrast, there was no significant main effect of postnatal treatment in the neuronal ratio within the Rh ($\beta = 4.17 \pm 2.33$, $t(19.69) = 1.79$, $p = 0.088$), suggesting that Rh neuronal ratio was not altered in AE animals. Importantly, the Re of SI animals had a lower neuronal ratio than Rh ($\beta = -4.29 \pm 1.66$, $t(11) = -2.59$, $p = 0.025$), an effect that was magnified in AE animals (see interaction, above).

Lastly, we examined the volume of each of the structures (fig. 1E). There was a significant interaction between postnatal treatment such that Re of AE animals were smaller than Re of SI controls ($\beta = -1.42 \times 10^8 \pm 4.84 \times 10^7$, $t(15.46) = -2.941$, $p = 0.003$). There was no main effect of postnatal treatment in the volume of Rh ($\beta = -5.86 \times 10^7 \pm 6.82 \times 10^7$, $t(12.77) = -0.860$, $p = 0.406$), indicating that the Rh of AE animals did not differ in size from Rh of SI controls. As an anatomical structure Re was significantly larger than Rh ($\beta = 3.63 \times 10^8 \pm 3.00 \times 10^7$, $t(9.15) = 12.096$, $p < 0.001$), although this difference was less pronounced in AE animals (see interaction indicating AE-induced $1.42 \times 10^8 \mu\text{m}$ reduction in Re volume, above), again indicating selective alteration to Re and not Rh.

Discussion

The results of our study demonstrate that early postnatal alcohol exposure causes persistent damage to nucleus reuniens of the ventral midline thalamus in the female rat. This damage manifests as a reduction in total neuron number and Re volume. Lack of alcohol-induced alterations in Rh, an adjacent midline thalamic nucleus, suggests that such damage is circuit-specific rather than a common outcome in the entire ventral midline thalamus.

We recognize that the current study exclusively examined the brains of female rats, preventing the ability to generalize the alcohol effect. However, this observation of very

significant (21%) neuronal loss that appears to selectively target reuniens within ventral midline thalamus establishes a strong need for rigorous examination of Re and the related mPFC-Re-HPC circuitry in both sexes following developmental alcohol exposure. Such need is emphasized by previous studies examining prefrontal and hippocampal alterations following early postnatal alcohol exposure in rats, which have been inconsistent in finding sex-related differences. While some literature did not find significant sex differences in molecular or anatomical measures within PFC or HPC [e.g., 5, 10], several behavioral studies have observed a differential influence of alcohol between sexes [e.g., 18].

A considerable body of work characterizes developmental alcohol exposure-related damage in HPC (summarized in [19]) and a small, but growing, body of literature indicates that mPFC is damaged as well [6, 20]. Prior to our study, the damage to the third component of mPFC-Re-HPC circuit — nucleus reuniens of the ventral midline thalamus — in a rodent model of third trimester alcohol exposure has never been reported.

Only one group has previously examined the impact of prenatal AE on corticothalamic connectivity [21, 22], but this research looked at alcohol exposure during a markedly different time point in development: gestational days 14-19, a rodent analogue of human second trimester. Although both studies [21, 22] observed reductions in corticothalamic connectivity following gestational alcohol exposure, they did not examine individual nuclei within ventral midline thalamus, and examined thalamic projections to somatosensory cortex. Thus, the current data provide the mechanism (loss of Re neurons) that impacts dysfunctional corticothalamic connectivity following developmental alcohol exposure. Together, the studies suggest that midline thalamic pathology is a key feature of alcohol-related brain damage in FASD, regardless of timing of alcohol exposure.

Human studies assessing the behavioral and neuroanatomical impact of prenatal alcohol exposure suggest that function within the frontal lobe [23], as well as structure and function in the HPC [24–26], correlate with behavioral abnormalities in FASD-afflicted individuals. Finally, one study reported reduced whole-thalamus volume in humans diagnosed with FASD [27].

Conclusion

The robust neuroanatomical alterations that we observed in thalamic nucleus reuniens represent a critical observation supporting the hypothesis that developmental alcohol exposure damages functional circuits to produce deficits in learning and executive function observed in FASD. Whether Re is targeted by ethanol directly or indirectly, via the connections to the structures (HPC, mPFC) that are particularly vulnerable to the effects of ethanol during that developmental period, remains to be established. We believe that our preliminary evidence of region-specific ventral midline thalamic damage in FASD will provide an overdue foundation to examine alterations in connectivity of the complex and heterogeneous thalamus.

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Fig. 1.

Influence of early postnatal alcohol exposure on ventral midline thalamic nuclei. (A) Confocal micrograph of immunofluorescently labeled tissue (blue = Hoechst, red = NeuN) with representative contours of thalamic nucleus reuniens (Re) and rhomboid nucleus (Rh). Alcohol exposure on postnatal days (PD) 4-9 resulted in reuniens-specific decreases in neuron number (B), neuron ratio (D), and volume (E) — but no changes in non-neuron number (C) — when female rats were examined in adulthood (PD72). All graphs illustrate individual data points, with bars representing mean $\pm$ SEM. *** p < 0.001, ** p < 0.010, * p < 0.050

Table 1:

Mean Weight and blood alcohol content

Postnatal treatment (n)	BAC on PD4 (mg/dL)	Weight on PD4 (g)	Weight on PD5 (g)	Weight on PD6 (g)	Weight on PD7 (g)	Weight on PD8 (g)	Weight on PD9 (g)	Weight on PD72 (g)
SI (8)		10.3 ± 0.3	11.9 ± 0.3	13.6 ± 0.4	15.3 ± 0.5	17.2 ± 0.5	19.2 ± 0.6	231.6 ± 7.2
AE (5)	378.6 ± 37.6	11.2 ± 0.2	11.4 ± 0.3	12.1 ± 0.4	13.5 ± 0.5	15.4 ± 0.4	17.2 ± 0.5	255.2 ± 22.2

All values are expressed as mean ± SEM.