Effects of Moderate Prenatal Alcohol Exposure on NMDARs expression and functionality in orbitofrontal cortex pyramidal neurons.

V. Licheri[1], J. Chandrasekaran[1], C.W. Bird[1], C. F. Valenzuela[1,2] & J.L. Brigman[1,2]

[1]Department of Neurosciences, University of New Mexico School of Medicine, Albuquerque, NM 87131, USA.
[2]New Mexico Alcohol Research Center, UNM Health Sciences Center, Albuquerque NM 87131, USA.

Introduction

Cognitive impairments associated with Fetal Alcohol Spectrum Disorders (FASD) include abnormalities in learning and memory, executive control and social behaviors.[1]

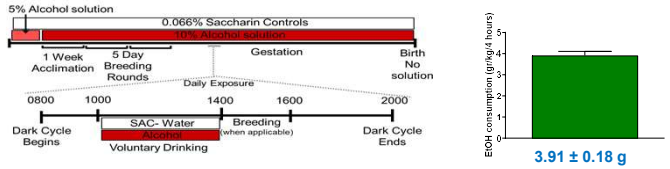
Our previous data shown that moderate prenatal alcohol exposure (**PAE**) impairs reversal learning and leads to alterations in coherence in the orbitofrontal cortex (**OFC**) and dorsal striatum (**dS**)[2,3].

Also, similar deficits are found when GluN2B subunit containing N-Methyl-D-Aspartate receptors (NMDARs) are knocked-down in cortex[4-6].

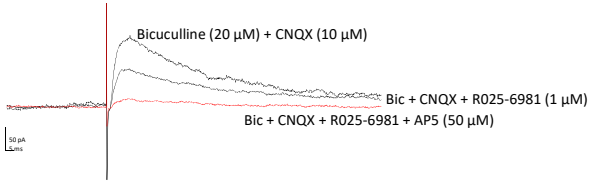
Considering recent findings showing that the GluN2A/GluN2B ratio plays an important role in the control of synaptic plasticity to support learning[7,8], we assume that GluN2B could be a possible molecular target involved in reversal learning deficits observed in PAE mice.

For this purpose, here we examined the GluN2B function and expression integrating whole cell patch clamp recordings and western blotting analysis using non training adult PAE mice (PND 90-100).

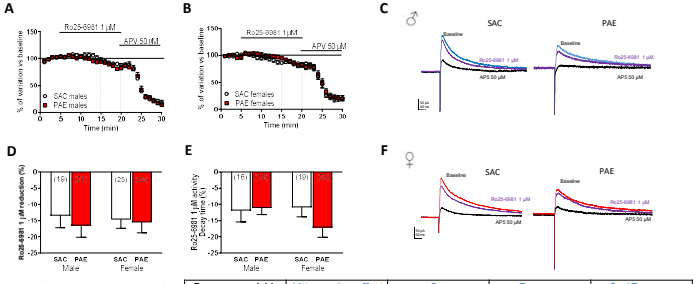
Prenatal Alcohol Exposure Paradigm



Pharmacological isolation of evoked NMDA-EPSCs



Synaptic evoked *EPSCs mediated by GluN2B-NMDARs in SAC and PAE mice.*



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Response variable	Litter random effect	Sex effect	Exposure effect	Sex*Exposure effect
Amplitude after Ro25-6981	Excluded	p = 0.925	p = 0.602	p = 0.785
Decay Time after Ro25-6981	Excluded	p = 0.260	p = 0.287	p = 0.180

aCSF: 2mM $CaCl_2$ - 1 mM $MgSO_4$ - Bicuculline 20 µM - NBQX 10 µM.

Conclusions

The Linear Mixed Model (LMM) analysis of NMDA-EPSC amplitude shows a significant increase in PAE female mice. While, in the group of PAE male mice we observe a significant reduction in current density.

Decay time of NMDA currents is not altered in PAE exposed mice from either sex.

PAE may induce alterations in the post-synaptic terminals of OFC pyramidal neurons in adult offspring, and these effects seem to be sex-specific.

Pharmacological isolation of GluN2B mediated currents revealed that PAE exposure does not modulate the GluN2B function in all experimental groups. This data is confirmed by western blotting, since we did not observe a significant change in synaptic expression.

Preliminary data about GluN2A functionality suggest that PAE exposure may modulate in positive manner the GluN2A activity in females.

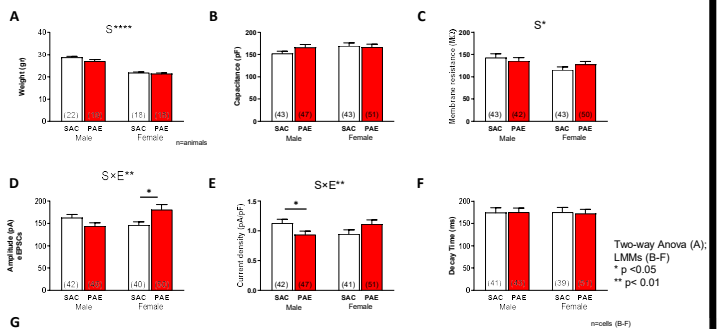
Further experimental investigations are needed to characterize the functionality and expression of extra-synaptic NMDARs in this PAE model.

These data demonstrate that the NMDARs are significantly modulated by PAE in OFC pyramidal neurons.

Materials and Methods

Moderate Prenatal Alcohol Exposure. Female C57BL/6J (Jackson Laboratories) mice underwent a limited access PAE paradigm. Throughout gestation, dams had access to 10% alcohol saccharin sweetened solution for four hours a day.
SAC and PAE offspring were housed in groupings of 2/cage in a temperature- and humidity-controlled vivarium under a reverse 12hr light/dark (lights off 0800hr) and tested during the dark phase. All experimental procedures were approved by the UNM Health Sciences Center Institutional Animal Care and Use Committee.
Western blotting for protein expression. OFC area was micro-punched from coronal slices and frozen immediately on dry ice. Tissue was homogenized by sonication in protease and phosphatase inhibitors (Sigma protease inhibitor cocktail and phosphatase inhibitor cocktails 1 and 2, 10 µM NaF, 1% Triton-X 100, 25 mM Tris, pH 6.8) by sonication and protein concentration was determined by the fluorescence-based quantification using the Qubit system. Immunoblotting was performed using the following antibodies- anti-NR2B(1:750, Cell Signaling), anti NR1(1:1500, Cell Signaling), then membrane was incubated in a secondary antibody, goat anti-rabbit IRDye 680RD for 45 minutes (infra-red fluorescence-based secondary antibody, LI-COR). Immuno-reactivity was detected by scanning with LI-COR Odyssey imager.
Preparation of mouse brain slices. After deep anesthesia with ketamine (250 mg/kg intraperitoneally) and transcardially perfusion, the brain was rapidly removed from the skull and transferred to NMDG cutting solution containing in (mM): 92 NMDG, 2.5 KCl, 10 MgSO4, 0.5 CaCl2, 1.25 NaH2PO4, 30 NaHCO3, 20 Hepes, 25 Glucose, 2 Thiourea, 3 Na-Pyruvate, 5 mM Na-ascorbate. Titrate pH to 7.3–7.4 with HCl. Coronal OFC slices 300 µm thick were cut using a Leica VT1000, and incubated for 30 minutes at 34 °C. During this incubation, the Na+ spike-in solution 2 M is added. The slices were then transferred to a chamber in Hepes holding aCSF solution containing in (mM): 92 NaCl, 2.5 KCl, 1.25 NaH2PO4, 30 NaHCO3, 25 glucose, 20 HEPES, 2 CaCl2, and 1 MgSO4. Titrate pH to 7.3-7.4 with a few drops of concentrated 10 NaOH.
Whole cell patch clamp recordings. A cesium chloride internal pipette solution in mM: 120 CsCl, 10 Hepes, 2 MgCl2, 1 EGTA, 2 MgATP, 0.3 mM NaGTP, 1 QX314, adjusted to 288 mOsm, pH 7.3-7.4 was used to recorded electrically-evoked excitatory postsynaptic currents (eEPSCs) in voltage clamp mode. A concentric bipolar stimulating electrode (FHC, USA) was placed in proximity to the recorded cell. Stimulus pulses were delivered every 20 sec (ranged from 10-1500 µA) to elicit a stable and submaximal evoked current. eEPSCs-NMDA were evoked at a holding potential of +40 mV and in presence of bicuculline (20 µM), and NBQX (10 µM). GluN2A and GluN2B currents were isolated pharmacologically using PEAQX (1 µM) and Ro25-6981 (1 µM). The NMDA receptor component was verified applying NMDAR-antagonist (APV 50 µM). Drugs used were purchased from Tocris and Hello Bio. The NMDA current mediated by specific subunits was measured as the difference between the current recorded during the last 5 minutes of bath-application vs baseline.
Statistical analysis. Data are expressed as means ± SEM and as percentage of variation ± SEM. Results (protein expression and weights) were compared by Two-way Anova using Prism software (version 9.0.1, GraphPad). While, Linear Mixed Models (SPSS vers. 26, IBM) were used for ex-vivo analysis because whole-cell patch clamp recordings were performed in animals coming from same litters. The model was built considering litter as a random effect, and sex and exposure indicated like a fixed effect. Rout test was used to detect possible outliers. A P value < 0.05, P < 0.01, P < 0.001 were considered statistically significant.

PAE induces sex-specific effects on synaptic evoked *NMDA-EPSCs in OFC pyramidal neurons.*



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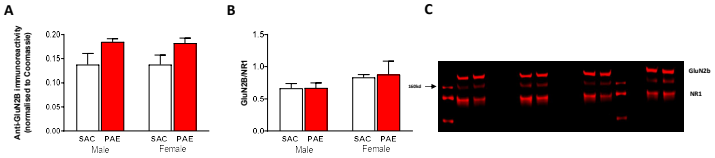
Response variable	Litter random effect	Sex effect	Exposure effect	Sex*Exposure effect
Capacitance	Excluded	p = 0.147	p = 0.363	p = 0.172
Membrane Resistance	Excluded	**p = 0.018**	p = 0.730	p = 0.192
Current Density	Excluded	p = 0.976	p = 0.871	**p = 0.009**
Males	Excluded	-	**p = 0.042**	-
Amplitude	Excluded	p = 0.413	p = 0.439	**p = 0.004**
Females	Excluded	-	**p = 0.019**	-
Decay Time	Included	p = 0.414	p = 0.849	p = 0.800

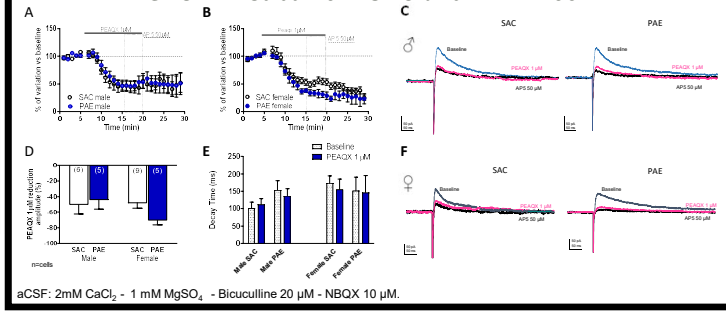
aCSF: 2mM $CaCl_2$ - 1 mM $MgSO_4$ - Bicuculline 20 µM - NBQX 10 µM.

PAE does not alter GluN2B-NMDARs expression.



n=2/group (8 mice, 4 mice pooled /group). Statistical analysis: Two-way ANOVA.

PRELIMINARY DATA: evoked NMDA-EPSCs mediated by GLUN2A subunit in SAC and PAE mice.



aCSF: 2mM $CaCl_2$ - 1 mM $MgSO_4$ - Bicuculline 20 µM - NBQX 10 µM.

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Acknowledgements

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