Respiratory vulnerability and brainstem damage induced by early ethanol exposure during intermittent hypoxia in rats, with selective neuroprotection by fish oil

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Materials and methods

fR and apneas in pretreatment phase. On PDs 3, 5 and 7 pups were removed from their maternal cage. One male and one female were placed in holding cages, partially filled with clean wood shavings. The floor of the cage was maintained at 37 ◦C (± 1 ◦C) using a heating pad. Pups were maintained in pairs to avoid undesirable effects of social isolation conditions [1, 2]. Fifteen minutes later, pups were weighed and randomly administered with 0.0 or 2.0 g/kg EtOH, via intragastric intubation (i.g.), as was described in[3]. The 2.0 g/kg EtOH dose was chosen because it was the minimum dose with a clear depressant effect upon respiration without altering pup's body weights (BW) [4]. The 2.0 g/kg EtOH dose was achieved by administering 0.015 ml/g of BW of a 16.8 % v/v EtOH solution (96 % proof alcohol, Porta Hermanos, Cordoba, Argentina). Similar volumes of distilled water were utilized in the case of pups administered with the dose of 0.0 g/kg. Pups were then returned to holding cages where they remained 15 additional minutes before being individually tested in the plethysmographic chambers (Pleth) in a whole-body plethysmograph (Plethysmograph Model 10 G equipped with the software "Breath Medidor de Respiracion", Itcom, Argentina) [4–7]. After 1 min acclimatization period in the chamber, fR and apneas (as the interruption of air flow for at least two normal respiratory cycles [8] were recorded in unrestrained awake pups during 5 min. The 5-min of the test was averaged, and it was defined as 1 bin. To avoid

neonatal thermal disruptions, the temperature inside the Pleth was kept at 31–32 ◦C (like their maternal nest thermal condition) using heating pads placed under the chambers.

C3/Hoechst staining and quantitative analysis. Coronal brain sections (thickness: 40 µm) were permeabilized for 10 min in PB 0.1 M containing 0.3 % Triton-X-100 (Sigma Chemical Co., St. Louis, MO, USA) diluted in PB 0.1 M. Sections were washed with PB 0.01 M (3 x 5 min) and treated with a blocking solution (5 % bovine serum albumin (BSA)-Sigma Aldrich, Merck, Germany) for 1 h at RT. Then, sections were incubated overnight at RT in constant and slow agitation with a polyclonal rabbit anti-caspase 3 antibody (Cell Signaling Technology; 1:750) and 2.5 % BSA. Slices were washed in PB 0.01 M (3 x 5 min) and incubated for 1 h at RT in darkness and constant agitation with fluorescent-labeled goat anti-rabbit secondary antibody (Alexa Fluor 488, ThermoFisher Scientific; 1:750) in PBS 0.1 M. Slices were stained with Hoechst (emission wavelength: 454 nm, Sigma Aldrich; 1:800) in PBS 0.1 M by 15 min and washed 2 x 5 min in PB 0.01 M. After washing, slices were mounted on coverslip with MOWIOL mounting media (Sigma Aldrich, Merck, Germany). For the C3 quantifications, C3 stained cells were detected in a fluorescent microscope (Zeiss Primo Star ILED) equipped with a 40x objective. Images were acquired with a high-resolution Zeiss Axiocam 208 videocamera and saved with ZEISS ZEN Program, version 3.8. It was quantified the total number of C3+ cells per field (image size 1920 x 1080 px) using Adobe Photoshop 2022. The researcher was blind relative to the specific treatment that each pup was exposed to. The images with the double C3/Hoechst labelling were detected in a confocal microscope Zeiss LSM 800 Airyscan equipped with a 20x objective and acquired with a high-resolution Zeiss Axiocam 506 videocamera and saved also with ZEISS ZEN Program, version 3.8. In these images, the field was divided into 9 sections of equal size and the number of pyknotic cells labeled with double C3/Hoechst stain were quantified in only 4 chosen areas.

Cytoarchitectural identification. Areas of interest (ROb, RMg, RPa and NTS) were identified and delimited according to the atlas by Paxinos & Watson (2007). The interval distance through Bregma of the RPa ranged from -14.30 mm to -9.80 mm; the interval of the ROb ranged from -14.30 mm to -11.30 mm; the interval of the RMg ranged from -12.00 mm to -9.72 mm; and the interval of the NTS ranged from -13.24 mm to -14.08 mm. **Figure 1S** illustrates representative areas of each nucleus of interest with the corresponding coordinates from Bregma.

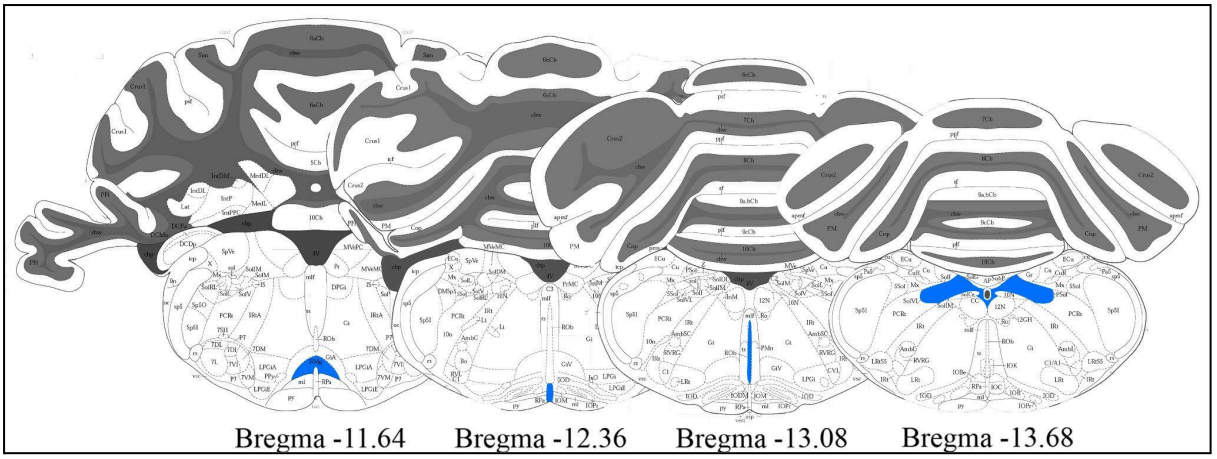

Figure 1S - Schematic drawing of coronal sections of the rat brain based upon the atlas of Paxinos and Watson (2007), showing representative areas of RMg, RPa, ROb and NTS. The anteroposterior (AP) coordinates from Bregma of sections included for detailed analysis were AP −11.64 (RMg); AP −12.36 (RPa); AP −13.08 (ROb); AP −13.68 (NTS). RMg = raphe *magnus*; ROb = raphe *obscurus*; RPa = raphe *pallidus*; NTS = nucleus of the solitary tract.

Results

Experiment 1: Body weights on PDs 3, 5, 7 and 9 as a function of early EtOH exposure. Repeated measures ANOVA (EtOH treatment × PD) indicated that BW were significantly affected by age [$F(3, 120) = 809.35$, $p < 0.0001$]. As expected, BW increased significantly in each PD compared to the other one. In this set of animals, EtOH treatments were not found to exert significant main effects or interactions **(Table S1)**.

Table S1. Body weight as a function of EtOH treatment and postnatal day.

Postnatal Day	Body weight (g)	
	Water (n = 21)	**EtOH (n= 21)**
PD 3	7.14 ± 0.14	7.41 ± 0.14
PD 5	9.03 ± 0.20***	9.40 ± 0.20***
PD 7	11.19 ± 0.27***	11.67 ± 0.27***
PD 9	13.40 ± 0.38***	14.07 ± 0.38***

$^{(***)}$ Significant differences of each PD compared to the other ones, $p < 0.001$.
Values are expressed as mean ± SEM.

Experiment 1: fR and apneas in pretreatment phase. The within-between ANOVA revealed significant effects of both main factors (EtOH treatment [F (1, 40) = 5.86, p = 0.0201] and PD [F (2, 80) = 20.61, p < 0.0001]). A significant interaction between the two factors was also observed [F (2, 80) = 5.18, p = 0.0077]. As expected, both EtOH- and water-exposed neonates exhibited a maturational normal increase in their fRs through PDs that achieved statistical significance at PD 7 compared to those observed at PD 3 and 5 **(Table S2)**. Yet, the EtOH intoxication started to exert the depressant effect on breathing in pups of 5 days-old, depression that was statistically significant at PD 7.

When considering the normalized total number of apneas as a function of EtOH treatment through PDs, ANOVA indicated a significant effect of the main factor PD [F (2, 80) = 24.52, p < 0.0001]. Fisher's post-hoc tests revealed that apneas decreased significantly in PD 7 **(Table S2)**. EtOH treatments were not found to exert significant main effects or interactions.

Table S2. Respiratory frequencies (fRs), absolute number of apneas (abs) and normalized number of total apneas (log) as a function of EtOH treatment and postnatal day.

Postnatal Day		Treatment	
		Water (n = 21)	**EtOH (n = 21)**
PD 3	**fRs**	167.83 ± 8.05	167.92 ± 8.25
	Apneas (abs)	29 (14 - 72)	34 (16 - 48)
	Apneas (log)	3.49 ± 0.26	3.52 ± 0.28
PD 5	**fRs**	171.57 ± 8.42	155.44 ± 8.63
	Apneas (abs)	59 (27 - 115)	50 (18 - 76)
	Apneas (log)	3.86 ± 0.24	3.89 ± 0.25
PD 7	**fRs**	232.46 ± 9.63 **	184.00 ± 9.86 ** ++
	Apneas (abs)	8 (0 - 21)	8 (3 - 32)
	Apneas (log)	1.86 ± 0.31 ###	2.47 ± 0.32 ###

(**) Significant differences of PD 7 vs PD 3 and 5 in Water-exposed neonates and only vs PD 5 in EtOH-exposed neonates compared to the other ones, p < 0.01. (++) Significant differences between EtOH- vs Water-exposed neonates, p < 0.01. (###) Significant differences of PD 7 vs PD 3 and 5, p < 0.001. Values are expressed as mean ± SEM or as median (95% confidence interval, CI), depending on whether the variable followed a normal distribution.

Table S3. Absolute number of apneas as a function of EtOH treatment, air condition and postnatal day at evaluation phase (PD 9).

Bin at the test	Treatment			
	Normoxia (n = 19)		HIS (n= 23)	
	Water (n = 9)	EtOH (n = 10)	Water (n = 12)	EtOH (n = 11)
B1	6 (0 - 7)	11 (0 - 20)	2 (0 - 20)	6 (1 - 47)
B2	14 (2 - 25)	7 (2 - 64)	3 (2 - 53)	4 (2 - 16)
B3	13 (1 - 18)	26 (5 - 68)	6 (2 - 44)	4 (0 - 21)
B4	17 (9 - 24)	12 (6 - 16)	3 (0 - 23)	2 (1 - 6)
B5	17 (6 - 48)	16 (10 - 22)	31 (7 - 64)	12 (2 - 21)
B6	19 (15 - 36)	12 (8 - 20)	4 (2 - 21)	3 (0 - 5)
B7	14 (8 - 22)	17 (7 - 34)	44 (20 - 106)	29 (1 - 38)

Values are expressed as median (95 % confidence interval, CI).

Experiment 2: Body weights at PDs 3, 5, 7 and 9 as a function of early EtOH exposure and fish oil administration.

Repeated measures ANOVA (EtOH treatment × Oil administration × PD) indicated that BW were significantly affected by age [$F(3, 234) = 1506.3$, $p < 0.0001$]. A significant interaction between EtOH treatment × PD was also observed [$F(3, 234) = 5.90$, $p = 0.0007$]. As expected, BW increased significantly in each PD compared to the previous one **(Table S4)**. In this set of animals, it was observed that EtOH-treated pups had lower BW than Water-treated at PD 9. However, this difference was very little and was observed mainly in pups treated with corn oil. No significant differences were found as a function of the oil administered across PDs.

Table S4. Body weight as a function of postnatal day, oil administration and EtOH treatment.

Postnatal Day	Body weight (g)			
	Water (n = 40)		EtOH (n= 42)	
	Corn oil (n = 18)	Fish oil (n = 22)	Corn oil (n = 20)	Fish oil (n = 22)
PD 3	7.32 ± 0.16	7.69 ± 0.15	7.67 ± 0.16	7.77 ± 0.15
PD 5	9.28 ± 0.25***	9.64 ± 0.22***	9.12 ± 0.23***	9.46 ± 0.22***
PD 7	11.59 ± 0.35***	12.05 ± 0.32***	11.21 ± 0.34***	11.64 ± 0.32***
PD 9	14.05 ± 0.46***	14.66 ± 0.42***	13.39 ± 0.43$^{***###}$	14.05 ± 0.42$^{***###}$

$^{(***)}$ Significant differences of each PD compared to the other ones, $p < 0.001$. $^{(###)}$ Significant differences between EtOH- vs Water-exposed neonates, independently of the oil administered, $p < 0.001$. Values are expressed as mean ± SEM.

Table S5. Absolute number of total apneas as a function of EtOH treatment, air condition and postnatal day at evaluation phase (PD 9).

Bin at the test	Treatment			
	Normoxia (n = 42)		HIS (n = 40)	
	Water (n = 21)	EtOH (n = 21)	Water (n = 19)	EtOH (n = 21)
B1	3 (1 - 9)	6 (2 - 16)	1 (0 - 6)	8 (3 - 32)
B2	24 (6 - 60)	11 (6 - 17)	4 (0 - 6)	5 (2 - 7)
B3	17 (7 - 30)	8 (5 - 49)	10 (3 - 17)	3 (1 - 8)
B4	15 (9 - 23)	8 (7 - 50)	2 (0 - 5)	1 (0 - 3)
B5	14 (8 - 37)	8 (3 - 24)	16 (8 - 36)	2 (1 - 5)
B6	9 (6 - 29)	9 (4 - 16)	2 (0 - 3)	1 (0 - 2)
B7	22 (6 - 41)	8 (3 - 20)	13 (8 - 38)	3 (2 - 21)

Values are expressed as median (95 % confidence interval, CI).

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