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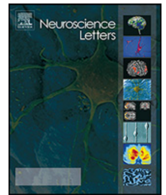

# Effects of maternal alcohol consumption during breastfeeding on motor and cerebellar Purkinje cells behavior in mice

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ABSTRACT

Purkinje cells (PCs) are the sole output from the cerebellar cortex. Their electrophysiological behavior may serve as indicator of chronic ethanol effects on the cerebellum. Here, we studied the effects of ethanol consumption through breastfeeding on motor behavior, histology and PCs electrophysiology. Mice with different maternal drinking regimen (ethanol, E or sucrose, S) during prenatal (E/and S/) and postnatal period (/E and/S) were compared. Motor performance in the runway and rotarod tests was significantly worse in mice exposed to ethanol prenatally (E/E and E/S) than in mice exposed to sucrose (S/S), with a limited influence, if any, of mother regimen during lactation (E/S vs E/E). A loss of 20–25% of PCs was found for both E/S and E/E compared to S/S mice but PC numbers were similar in S/E and S/S. Mean PC spontaneous simple spike firing rate and rhythmicity were higher in E/S and E/E than in S/S but there was no difference between S/E and S/S. Complex spike frequency was similar in all groups. In contrast, complex spike duration and the related pause induced on the simple spike firing were shorter in E/E and in E/S, but no difference was found between S/E and S/S. We conclude that cerebellar dysfunction induced by maternal ethanol consumption in mice depends upon the drinking regimen during pregnancy and not during lactation.

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Ethanol consumption during pregnancy is highly damaging for the fetus. It may cause pre- and postnatal growth retardation, central nervous system dysfunction and a typical facial dysmorphism, characterizing the fetal alcohol syndrome (FAS) [12,13]. Since ethanol drinking may occur after birth, it has been suggested that offspring may suffer from analogous toxicity if exposed to alcohol via maternal milk [17,18,21,24]. Here, we studied the effect of ethanol consumption during breastfeeding on motor behavior, histology and electrophysiology of cerebellar Purkinje cells (PCs) in a validated mouse model of FAS in which abnormal motor behavior and histological and electrophysiological alterations in cerebellum were characterized [28].

Mice were generated from of 3- to 5-month-old NMRI mice mothers drinking *ad libitum* either ethanol 18% or sucrose 25% in a water solution during the prenatal period (starting one day before mating until delivery) and/or postnatally (for 15 days following delivery) while breastfeeding [26,28]. This study was approved by local ethical committees.

Four mice groups were defined by maternal drinking regimen before and after delivery (Fig. 1): sucrose during pregnancy and lactation (S/S, $n = 44$), ethanol during pregnancy and lactation (E/E, $n = 28$), ethanol during pregnancy and sucrose during lactation (E/S, $n = 25$), and sucrose during pregnancy and ethanol during lactation (S/E, $n = 24$).

Behavioral studies were carried out from postnatal day (PND) 24 (Fig. 1). Motor coordination was examined with rotarod and runway tests as previously described [1,26]. Animals were given four trials per day during three consecutive days (PND24-26) and the third day, after their last trial, mice were tested by using the runway test. As in rotarod, animals were given four trials per day for three consecutive days (PND26-28). After a 3-week trial-free period mice were retested by using rotarod and runway tests with four trials for each test during the same day (PND49).

Electrophysiological and histological studies started from PND 35 (Fig. 1). Single unit recording in PC were carried out in alert mice as previously described [28].Recordings were performed in lobules IV–VIII with glass micropipettes filled with NaCl 0.2 M (1.5–5 MΩ

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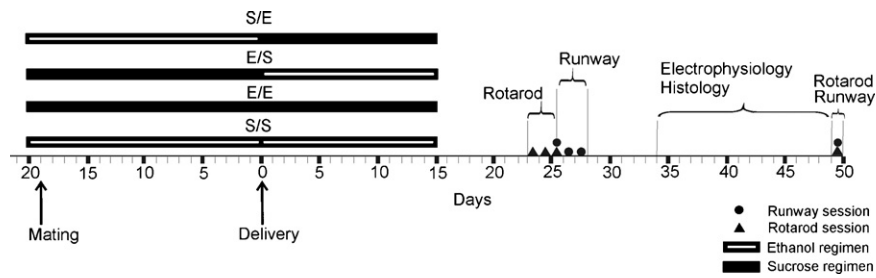


**Fig. 1.** Experimental schedule. Ethanol (E) and/or sucrose (S) in water solution were administrated pre- and/or postnatally determining the four groups (black and white rectangles for ethanol and sucrose administration periods respectively). Behavioral testing started at postnatal day 24. Electrophysiological and histological testing started at postnatal day 35. After a 3-week test-free period, repeat behavioral tests were performed.

of impedance). We used the same criteria for PC identification and data analysis (simple spike frequency, rhythmicity index, coefficient of variation, complex spike frequency, complex spike duration and pause) as in previous studies [6,28].

For the immunohistochemical staining, animals were anesthetized by i.p. injection of sodium pentobarbital (60 mg/kg) and transcardially perfused with 0.9% saline followed by 0.1 M phosphate-buffered 4% paraformaldehyde. The brain was dissected out, cryoprotected in 30% sucrose, and sectioned in 15 μm-thick slices. Sections were then labeled using an anti-calbindin antiserum. After a first incubation during 1 h with 10% normal horse serum (Hormonologie Laboratoire, Marloie, Belgium) and 0.1% Triton X-100 in PBS, sections were incubated 36 h at 4 °C with a polyclonal rabbit anti-calbindin-D28K antiserum (1/10000; Swant) in PBS and, successively with biotinylated donkey anti-rabbit IgG (H + L) (1/200; Jackson Laboratories, West Grove, PA) and ABC complex (Elite ABC kit; Vector Laboratories, Burlingame, CA). The peroxidase activity was revealed by diaminobenzidine in the presence of hydrogen peroxide. PCs were counted in cerebellar lobule X in three mediosagittal slices 100 μm-apart, in mice aged 5- or 7-week-old by an investigator blinded to the drinking regimen as previously described [28].

After confirming the normal distribution of the data using a Kolmogorov–Smirnov test, statistical comparisons among groups were performed using ANOVA one-way for repeated measures and Games–Howell post hoc test ($P > 0.05$) on the behavioral data (average values of the four trials per day per mice group) and ANOVA one-way and Tukey post hoc test ($P > 0.05$) for electrophysiological and histological data (STATISTICA 7.0 and SPSS Statistics 17.0.0). Results are expressed as mean ± standard deviation.

A higher mortality rate was observed in the offspring of mice receiving ethanol ($P < 0.05$, $P < 0.01$, $P < 0.001$ for E/E, E/S and S/E groups respectively compared to S/S). The mothers in E/S looked less after their pups. This observation was also made in mothers in the S/E group, i.e. mothers previously naïve to alcohol who were exposed to it after delivery. Suvivors' weight on PND 24 was 11.2 ±1.65 g for S/S, 9.0 ± 1.26 g ($P$ <0.001) for E/E, 11.7 ±3.06 g for E/S and 13.1 ± 0.71 g for S/E.

On the first day on rotarod test (Fig. 2A), mice whose mothers drunk alcohol during pregnancy (E/S, E/E) presented lower mean scores than controls (S/S) regardless of the postnatal regimen ($P < 0.0001$ for E/E and $P < 0.05$ for E/S). On the second day the mean values were still below controls' although significance level was not reached for E/E $P > 0.05$ (E/S $P < 0.05$). Interestingly, mice exposed to alcohol only after birth (S/E) obtained similar scores to S/S ($P > 0.05$) on both days. On the third day, all groups obtained similar scores, suggesting successful learning. After a 3-week test-free period, all groups presented mean scores below control values ($P < 0.0001$for E/S, $P < 0.05$ for S/E), though not significantly for mice exposed to alcohol during both pregnancy and lactation (E/E).

A similar pattern of performance across groups was observed in the runway test (Fig. 2B). E/S and E/E mice showed significant impairment compared to controls (first day $P < 0.0001$ for E/S and $P < 0.001$ for E/E; second day $P < 0.0001$ for E/S and $P < 0.05$ for E/E). On the second day E/S mice had significantly lower scores than E/E mice ($P < 0.0001$), whose scores were similar to S/E. On the third day, only E/S did not reach control values ($P < 0.0001$). After a 3-week test-free period, all groups demonstrated impairment compared to controls (S/S); this reached significance for E/S and S/E ($P < 0.001$ and $P < 0.05$ respectively) but not for E/E.

Overall performance of mice exposed to ethanol during pregnancy was significantly worse than that of mice exposed to sucrose, with a limited influence, if any, of mother regimen during lactation.

PC (S/S $n = 25$, E/E $n = 24$, E/S $n = 43$, S/E $n = 36$) were recorded in 19 mice (S/S $n = 4$, E/E $n = 6$, E/S $n = 4$, S/E $n = 5$). Mean spontaneous simple spike firing rate was significantly higher ($P < 0.001$) in E/S (92.87 ± 33.31 Hz) and E/E (105.90 ± 42.77 Hz) than in S/S (64.25 ± 24.67 Hz) (Fig. 3A). In contrast, there was no difference in firing rate between S/E (62.78 ± 27.89 Hz) and S/S. Similarly, simple spikes rhythmicity was enhanced in E/S (0.10 ± 0.12) and E/E (0.16 ± 0.22) compared to S/S (0.02 ± 0.01) ($P < 0.005$), whereas there was no significant difference in rhythmicity index between S/E (0.03 ± 0.06) and S/S mice (Fig. 3B). There were no signifi-

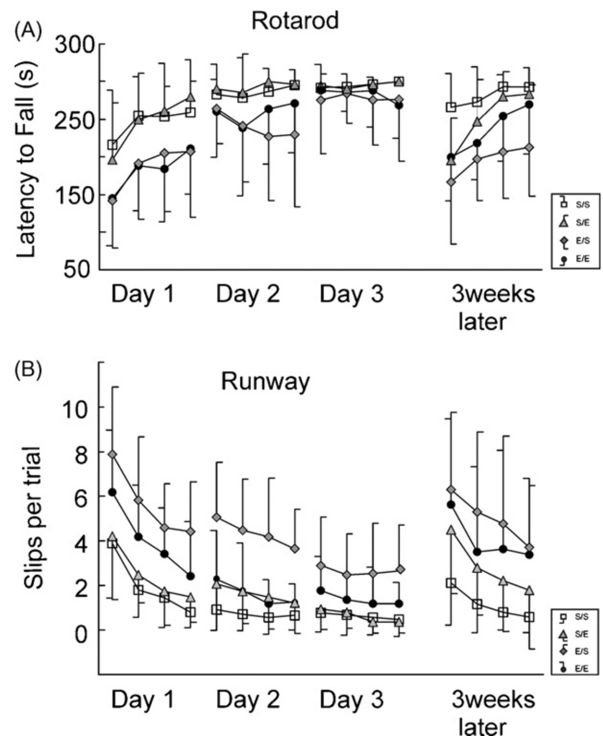


**Fig. 2.** Behavioral testing. (A) Latency to fall off the rotarod and number of slips from the runway bar (B) during the learning session (days 1–3) and after a 3-week test-free period.

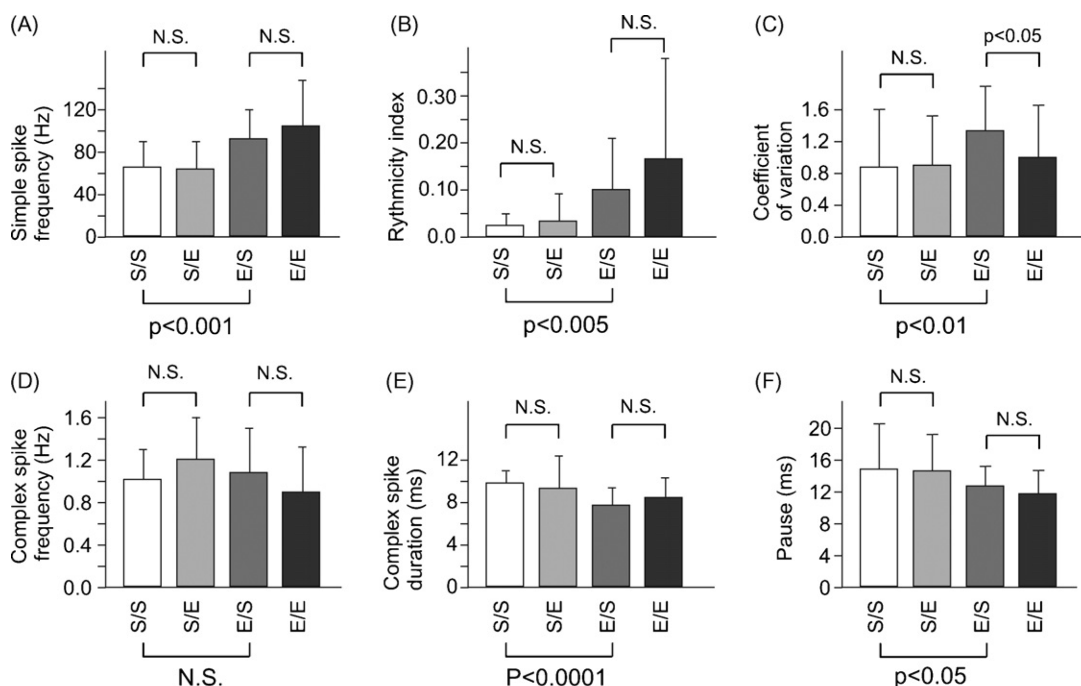


**Fig. 3.** Electrophysiological studies. Mean values ± standard deviation of (A) Simple spike frequency, (B) Rhythmicity index, (C) Coefficient of variation, (D) Complex spike frequency, (E) Complex spike duration and (F) Complex spike-related pause in simple spike firing, of Purkinje cells Recorded in E/E, E/S, S/S and S/E mice.

cant differences in complex spike firing rate between the different groups (Fig. 3D). However, complex spike duration was significantly lower in E/E (8.48 ± 1.83 ms) and E/S (7.73 ± 1.74 ms) mice than in S/S (9.83 ± 1.12 ms) ($P < 0.0001$) (Fig. 3E). The transient pause in the simple spike firing following a complex spike was also significantly reduced in E/E (11.91 ± 2.84 ms) and in E/S mice (12.41 ± 2.49 ms) ($P < 0.05$) compared to S/S (15.12 ± 6.12), while there was no difference between S/E (14.66 ± 4.89) and S/S (Fig. 3F). Finally the coefficient of variation of E/S mice (1.34 ± 0.53) was significant higher ($P < 0.01$) than controls' (0.86 ± 0.74) and also ($P < 0.05$) than E/E group (1.00 ± 0.69).

Overall, (1) the only significant difference in PC firing behavior between E/E and E/S mice group was the simple spike firing coefficient of variation for which E/S mice exhibited the highest value ($P < 0.05$) (Fig. 3C); (2) there was no difference in PC firing between S/S and S/E; (3) E/S and E/E exhibited the pattern of PC firing behavior previously described in FAS [28]. Thus ethanol significantly altered PC firing if administered prenatally (regardless of lactation regimen) but not if given only postnatally through breastfeeding.

PCs were counted in lobule X in three cerebellar mediosagittal slices distant for more than 100 μm from mice aged 5 or 7-week-old (S/S $n = 6$, E/E $n = 5$, E/S $n = 4$, S/E n = 4). There were 60.0 ± 11.3 PCs in S/S, 48.2 ± 11.5 PCs in E/E, 44.6 ± 13.8 PCs in E/S and 60.1 ± 11.4 PCs in S/E. This demonstrated a loss of 20–25% for both E/S and E/E compared to S/S mice ($P < 0.05$). There was no difference in the PCs number between S/E and S/S groups.

We found that behavioral, electrophysiological and histological alterations in cerebellum induced in the offspring by maternal ethanol consumption in mice depend upon the drinking regimen during pregnancy and not during lactation.

Mice prenatally exposed to alcohol showed a significant decrease of PC number [25] and severe alterations in surviving PC firing, including increased spontaneous simple spike firing rate and rhythmicity [28]. In contrast, mice exposed to alcohol through breastfeeding only showed no significant alteration in number or in bursting pattern of PC. This was reflected in motor behavior, as mice prenatally exposed to alcohol scored significantly worse whatever the maternal drinking regimen during lactation.

In our model, the dose of ethanol received by pups is not controlled and is difficult to estimate. Given high maternal stress in ethanol consuming female, leading to high mortality rate in offspring, repeated blood sampling of pups and/or mothers during the newborn period could not be performed. However, the importance of the alcoholization paradigm should be recognized as it may explain some variations in published results [30]. Many animal models of FAS involve massive alcohol doses mechanically delivered [14] at a specific short postnatal period (PN4-5) yielding into high BAC (3.5 g/l) [8,11,29]. Such doses produce significant and irreversible Purkinje and granular cell loss in cerebellum as well as markedly impaired motor behavior [3,9,19,20]. More realistic doses to BACs of 2.5 g/l also lead to significant deleterious, but less severe effects on cerebellum [9]. The doses used in our model, which is more ecological, lead to blood alcohol concentrations BACs of 0.73 g/l in mothers [28].

In vitro, postnatal alcohol exposure in mice affect the cerebellum at various levels, including GABAergic and glutamatergic transmission, and voltage-gated ion channels: It enhances GABAergic transmission to granule cells via an increase in Golgi cell excitability [4]. Alcohol also blocks long-term depression induction at climbing fiber-PC level [5] and at parallel fiber-PC level [2]. In vivo, chronic exposure to high dose ethanol (18% ad libitum) leads to mild decrement of simple spike firing and minor motor coordination impairment [26].

FAS mouse models have shown that low doses of alcohol may cause serious fetal damage. BAC of 0.65 g/l in mothers provoke a delay or failure of proper proliferation and migration of neuroepithelial cells into the midline of neural tube and impinge proper migration and development of serotonin neurons in the fetal brain [31]. In cerebellum of offspring, maternal BACs of 0.73 g/l was associated with significant PC loss and important dysfunctions of surviving PC, such as increased simple spike firing rate, rhythmicity and synchronicity, leading to the emergence of fast local field potential oscillation [28]. Increased simple spike firing rate and rhythmicity has been recorded in different models of ataxic mutant

animals [6,7] and in fetal alcohol syndrome [28] [26], but never in normal animals. The current hypothesis is that increased simple spike rhythmicity and synchronicity give rise to fast local field potential oscillation that 'traps' other PC in the same rhythmic firing pattern, making them less reactive to external stimulation [27].

During human and rat breastfeeding, alcohol concentrations in milk range between 44–80% of maternal blood concentrations [10]. Behavioral change has been noted in rodents exposed to low concentrations (1.75 g/l) of alcohol in maternal milk [24]. Ethanol can adversely influence maternal care of the offspring [23], which may have contributed to higher offspring mortality of all ethanol consuming groups. This may also partially explain the tendency of S/E to perform worse than control after a 3-week test-free period and the absence of difference between E/E and S/E in these tests. The contrasting effect of ethanol on pups' weight may be related to the high neonatal mortality rate in ethanol consuming groups, resulting in a selection of the strongest pups and increased milk/pup ratio.

Human babies are sensitive to alcohol in breast milk from less than two standard alcohol glasses consumed by the mother (BAC <0.5 g/l), resulting in changes in sleep cycles, suckling behavior and overall milk intake [17,18]. A study that took into account >100 potentially confounding variables showed that one alcohol drink per day consumed by mothers during the first 3-months of breastfeeding produces deleterious effects on motor development at one year of age but no effect on mental development [15]. However, the same group failed to reproduce these results in a comparable population of 18-month-old toddlers [16]. In our study, mother mice consumed an alcohol dose leading to a BAC of 0.73 g/l. In humans, this BAC may be reached by drinking 3 alcohol drinks (30 g ethanol).

We found histological, electrophysiological and behavioral alteration previously reported in this FAS model [28] only in offspring of ethanol-drinking mother during gestational period. Paradoxically, we observed that mice exposed to alcohol during fetal life as well as during the whole lactation period (E/E) obtained much better scores in the runway test than E/S. This difference remained even after a 3-week test-free period and may be related to withdrawal phenomenon. Notably, it has been shown that primary neuronal cultures of cerebellar cells presented enhanced intracellular $Ca^{++}$ signals after 1-day withdrawal period [22]. However, the difference reported in the runway performance was not present in the rotarod test and was not corroborated by either major electrophysiological nor anatomical difference in these mice. The only significant difference reported in PC physiology was the increase in coefficient of variation of simple spike firing in E/S, the mice subjected to withdrawal effect. This effect may be viewed as the expression of increased irregularity of these cells probably induced by a higher variability of cerebellar input during withdrawal. The motor effect could be thus associated to the negative effects of an alcohol withdrawal effect acting on the other part of the CNS outside the cerebellum.

In conclusion the present study shows that a validated mouse model producing serious cerebellar abnormalities associated with antenatal exposure to ethanol does not provide good evidence of cerebellar toxicity with ethanol exposure through breastfeeding.

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